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(54) Title: DIRECTED COMBINATORIAL COMPOUND LIBRARY AND HIGH THROUGHPUT ASSAYS FOR SCREENING SAME

(57) Abstract

A combinatorial library comprising a predetermined collection of nucleoside peptide molecules for inhibiting the transfer of a sugar from a selected sugar nucleotide donor to a selected acceptor by a carbohydrate processing enzyme. The nucleoside peptide molecule comprises (a) a nucleoside monomer; (b) a spacer monomer coupled to the nucleoside monomer wherein the spacer monomer comprises one or more amide linked amino acid residues, or a peptidomimetic; and (c) cap monomers attached to the spacer monomer. The nucleoside peptide molecules differ from each other as to the identity of at least one element of the nucleoside monomer, spacer monomer, or cap monomer.

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TITLE: DIRECTED COMBINATORIAL COMPOUND LIBRARY AND HIGH THROUGHPUT ASSAYS FOR SCREENING SAME

FIELD OF THE INVENTION

The invention is directed to predetermined libraries of compounds, related compounds useful for making such libraries, and compositions containing the compounds.

BACKGROUND OF THE INVENTION

Interactions between proteins and carbohydrates are involved in a wide array of biological recognition events, including fertilization, molecular targeting, intercellular recognition, and viral, bacterial, and fungal pathogenesis. The oligosaccharide portions of glycoproteins and glycolipids mediate recognition between cells and cells, between cells and ligands, between cells and the extracellular matrix, and between cells and pathogens.

Inhibition of carbohydrate processing enzymes involved in the synthesis, transport, and cleavage of oligosaccharides can be used as a means to inhibit interactions between proteins and oligosaccharides and inhibit the recognition phenomena. In particular, two groups of enzymes associated with the *in vivo* synthesis of oligosaccharides can be targeted. The enzymes of the Leloir pathway transfer sugars activated as sugar nucleoside phosphates to a growing oligosaccharide chain. The nucleoside phosphate building blocks involved in the Leloir pathway include: UDP-Glc, UDP-GlcUA, UDP-GlcNAc, UDP-Gal, UDP-GalNAc, UDP-Idua, GDP-Man, GDP-Fuc, and CMP-NeuAc. The other group of enzymes associated with the *in vivo* synthesis of oligosaccharides are the non-Leloir pathway enzymes that transfer carbohydrate units activated as sugar phosphates, but not as sugar nucleoside phosphates.

Glycosyltransferases catalyze the addition of activated sugars from nucleotides in a stepwise fashion to a protein or lipid or to the non-reducing end of a growing oligosaccharide. There are estimated to be over 200 glycosyltransferases encoded by mammalian cells, many of which appear to be developmentally regulated, resulting in tissue specific-patterns of glycosylation (Schachter, H. *Curr.Opin.Struct.Biol.* 1:755-765, 1991; and Paulson, J.C. and Colley, K.J. *J.Biol.Chem.* 264:17615-17618, 1989). Each NDP-sugar residue requires a distinct class of glycosyltransferase and each of the glycosyltransferases appear to catalyze the formation of a unique glycosidic linkage. Oligosaccharides may be linked to proteins by N-glycosidic or O-glycosidic linkages. In an N-linkage, an N-acetyl glucosamine residue is β-linked to the amide nitrogen of an Asn in the sequence Asn-X-Ser or Asn-X-Thr (X is any amino acid). In an O-linkage, the disaccharide β-galactosyl-(1,3)-alpha-N-acetylgalactosamine is alpha-linked to the hydroxyl group of serine or threonine.

The Golgi enzymes β(T1-6) *N*-acetylglucosaminyltransferase V (i.e. GlcNAc-TV) and core 2 β(T1-6) *N*-acetylglucosaminyltransferase (i.e. core 2 GlcNAc-T) are responsible for the extension of GlcNAc β(T1-6) branched *N*- and *O*-linked carbohydrate side chains of cell-surface glycoproteins. These side chains are found on the surface of human tumor cells and they have been associated with cancer invasion and metastasis (Dennis *et. al.*, *Science 236*: 582, 1987; Demetriou *et. al.*, *J. Cell Biol. 130*:383, 1995). GlcNAc-TV and core 2 GlcNAc-T have been shown to be up-regulated in human carcinomas (Fernandes et al., Cancer Res. 51:718-723, 1991; Shimodaira, K. et al, Cancer Research

57:5201, 1997), a phenomenon that has been associated with the activation of the *ras* signaling pathway (Dennis et al., Science 236:582-585, 1987; Dennis et al Oncogene 4:853-860, 1989)). Overexpression of GlcNAc-TV in epithelial cells has been found to result in morphological transformation and tumor formation in mice (Demetriou et al, J. Cell Biol. 130:383-392, 1995). Therefore, GlcNAc-TV as well as enzymes supplying acceptor substrates to GlcNAc-TV (i.e. GlcNAc-TI, α-mannosidase II and core 2 GlcNAc-T of the *O*-linked pathway) are useful targets for anti-cancer pharmaceuticals.

Fucosyltransferases are involved in determining the expression of sialyl Lewis^x (sLex^X) antigen on the surface of blood cells. In the inflammatory process, selectin-sLex^X mediated attachment of leukocytes is a key step for leukocyte activation and trans-endothelial migration. Inhibition of fuscosyltransferases responsible for the synthesis of sLex^X will prevent the formation of selectin-carbohydrate complexes and therefore will interfere with the first step of the inflammatory process. Inhibitors would be useful for the treatment of chronic inflammatory disorders such as asthma, rheumatoid arthritis, inflammatory bowel disease and atherosclerosis. All of these disorders are conditions where an inappropriate inflammatory response is involved and suppression is desirable.

Blocking of certain enzymes in the carbohydrate processing pathway leads to an increased sensitivity of immune cells to Th1 cytokines (interferon and interleukin-2), thereby further promoting the Th1 immune response. While interferon-alpha itself has anti-viral activity, it appears to be insufficient on its own in eliminating chronic infections such as hepatitis. Therefore, enzyme inhibitors can be used to enhance the effect of Th1 cytokines in the treatment of many viral, bacterial, fungal and parasitic infections, including hepatitis B and C.

Inhibitors of enzymes that synthesize specific carbohydrate structures of bacteria that play an important role in pathogenicity can be used to enhance the susceptibility of the bacteria to the host immune system and to inhibit the entry of the bacteria into human cells and tissues. For example, a specific bacterial carbohydrate structure called low molecular weight oligosaccharide (LOS) that is similar to a carbohydrate structure found on human glycoproteins and glycolipids, protects the bacterium from being recognized and cleared by the host's immune system. Inhibitors of the enzymes responsible for synthesizing the LOS structure can reduce the ability of bacteria such as *N. gonorrhea* to elude immune surveillance in a host.

It is apparent that there is a need for small molecule inhibitors of carbohydrate processing enzymes including GlcNAc-transferases I through V, galactosyltransferases, sialo transferases, fucosyl transferases, and core 2 GlcNAc, with structural and conformational diversity. There is also a need for high throughput methods for screening the inhibitors to identify "lead" pharmaceutical compounds.

SUMMARY OF THE INVENTION

The present invention utilizes a combinatorial chemistry approach. Combinatorial chemistry generally involves linking together, in step-wise fashion, identical or non-identical building blocks typically referred to as "monomeric units", or "chemical groups". Using this approach, the present inventors developed combinatorial libraries of small molecule inhibitors of carbohydrate processing

enzymes that transfer a sugar from a specific sugar nucleotide donor to a specific acceptor. The small molecule inhibitors have structural and conformational diversity. Enzymes that may be inhibited by the small molecule inhibitors include eukaryotic and procaryotic glycosyltransferases. The molecules can be screened using high throughput methods enabling identification of lead pharmaceutical compounds.

Broadly stated the present invention relates to a combinatorial library comprising a predetermined collection of nucleoside peptide molecules for inhibiting the transfer of a sugar from a selected sugar nucleotide donor to a selected acceptor by a carbohydrate processing enzyme wherein a nucleoside peptide molecule comprises (a) a nucleoside monomer; (b) a spacer monomer coupled to the nucleoside monomer wherein the spacer monomer comprises one or more amino acids, or a peptidomimetic or peptide analog; and (c) cap monomers attached to the spacer monomer; wherein the nucleoside peptide molecules differ from each other as to the identity of at least one element of the nucleoside monomer, spacer monomer or cap monomer.

In addition, a nucleoside peptide molecule is contemplated comprising (a) a nucleoside monomer; (b) a spacer monomer coupled to a nucleoside monomer, wherein the spacer monomer comprises one or more amino acids, or a peptidomimetic or peptide analog; and (c) cap monomers attached to the spacer monomer.

The invention also relates to a process for preparing a combinatorial library containing a predetermined collection of nucleoside peptide molecules for inhibiting the transfer of a sugar from a selected sugar nucleotide donor having a heterocyclic amine base, to a selected acceptor by a carbohydrate processing enzyme comprising:

(a) coupling one or more amino acids or a peptidomimetic or peptide analog, to a nucleoside monomer unit which nucleoside monomer unit comprises a heterocyclic amine base coupled to a sugar wherein the base corresponds to the heterocyclic amine base of the sugar nucleotide donor, or a modified form or analogue of the base; and

(b) capping any free functional groups or amine groups with a cap monomer unit.

The invention also relates to methods of using the combinatorial library for screening for pharmacologically active molecules; and pharmaceutical compositions containing compounds identified by the methods.

Further, the invention contemplates a solid-phase bioassay for identifying a compound having inhibitory activity against a carbohydrate processing enzyme which comprises (a) coupling an acceptor for the carbohydrate processing enzyme to a polymer and coating onto a carrier; (b) adding a carbohydrate processing enzyme, a sugar nucleotide donor labeled with a detectable substance, and a test compound; and (c) measuring the detectable change produced by the detectable substance.

The invention also contemplates a method for identifying a compound that inhibits N-linked oligosaccharide processing comprising (a) reacting a test compound with cells expressing N-linked oligosaccharides, in the presence of leukoagglutinating phytohemagglutinin (L-PHA) and measuring alkaline phosphatase activity; and (b) comparing to a control in the absence of the compound wherein an increase in alkaline phosphatase activity indicates that the compound inhibits N-linked oligosaccharide processing. The method may be used to identify compounds that inhibit all steps in the

N-linked oligosaccharide pathway prior to β1-4 Gal-transferase, including compounds that inhibit the carbohydrate processing enzymes specifically described herein, and Golgi α-mannosidase.

A compound contemplated by the present invention having inhibitory activity against a carbohydrate processing enzyme can be useful for the treatment and prophylaxis of tumor growth and metastasis of tumors; the prevention of tumor recurrence after surgery; the treatment of other anti-proliferative conditions such as viral infections; the stimulation of bone marrow cell proliferation, the treatment of immunocompromised patients, such as patients infected with HIV, or other viruses or infectious agents including bacteria and fungi; the prevention and treatment of diseases caused by bacterial pathogens having carbohydrate structures on their surface associated with virulence such as *Neisseria, Haemophilus, E. coli, Bacillus, Salmonella, Campylobacter, Klebsiella, Pseudomonas, Streptococcus, Chlamydia, Borrelia, Coxiella, Helicobacter*, and *Mycobacterim* species; or, the treatment of inflammatory disorders such as asthma, rheumatoid arthritis, inflammatory bowel disease, and atherosclerosis. A compound of the invention may also be used in patients undergoing bone marrow transplants, and as hemorestorative or chemoprotective agents in patients with chemical or tumor-induced immune suppression.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described in relation to the drawings in which:

Figure 1 is a schematic diagram showing a process for preparing 2,3-*O*-isopropylidineuridine from uridine;

Figure 2 is a schematic diagram showing a process for preparing 2,3-O-isopropylidene-5-O-methane sulfonyl uridine from 2,3-O-isopropylidine uridine;

Figure 3 is a schematic diagram showing a process for preparing 5-deoxy-5-azido-2,3-O-isopropylidenyl uridine from 2,3-O-isopropylidene-5-O-methanesulfonyl uridine;

Figure 4 is a schematic diagram showing a process for preparing 5-deoxy-5-amino-2,3-O-isopropylidenyl uridine from the parent azide;

Figure 5 is a schematic diagram showing a process for coupling an N-Boc protected spacer monomer unit to a nucleoside monomer unit;

Figure 6 is a schematic diagram for a process for deprotecting an N-Boc protected spacer monomer unit coupled to a nucleoside monomer unit;

Figure 7 is a schematic diagram showing a process for repeated coupling of Fmoc –protected spacer monomer units to a nucleoside monomer unit;

Figure 8 is a schematic diagram for capping a spacer monomer unit that is coupled to a nucleoside monomer unit;

Figure 9 is a schematic diagram showing the synthesis of a glycopolymer for a solid-phase core 2 GlcNAc-T assay;

Figure 10 is a schematic diagram showing a glycopolymer for a solid-phase GlcNAc-T V assay;

Figure 11 is a graph showing the distribution of normalized core 2 GlcNAc-T assay results for 1600 assays expressed as % control; and

Figure 12 is a graph showing the results of a high-throughput screen to detect microbial extracts with inhibitory effects on N-linked oligosaccharide processing in MDAY-D2 cells.

DETAILED DESCRIPTION OF THE INVENTION

Nucleoside Peptide Molecules

As used herein "monomer unit" refers to a molecule prior to coupling or conjugation to another monomer unit. A "monomer" refers to a molecule after coupling or conjugating to form a nucleoside peptide molecule. Monomer units used in the present invention to form the predetermined nucleoside peptide molecules include a nucleoside monomer unit, a spacer monomer unit, and a cap monomer unit.

A nucleoside monomer unit is selected based on the type of carbohydrate processing enzyme targeted for inhibition, and in particular the sugar nucleotide donor for the enzyme from which a sugar is transferred to an acceptor. A "sugar nucleotide donor" refers to a molecule comprising a nucleotide having a sugar component, a heterocyclic amine base, and a phosphate unit, coupled to a selected sugar that is transferred by a carbohydrate processing enzyme to an acceptor. An "acceptor" refers to the part of a carbohydrate structure (e.g. glycoprotein, glycolipid) where the selected sugar is transferred by a carbohydrate processing enzyme.

Carbohydrate processing enzymes for which combinatorial libraries may be prepared in accordance with the invention include eukaryotic glycosyltransferases involved in the biosynthesis of glycoproteins, glycolipids, glycosylphosphatidylinositols and other complex glycoconjugates, and prokaryotic glycosyltransferases involved in the synthesis of carbohydrate structures of bacteria and viruses, including enzymes involved in LOS and lipopolysaccharide biosynthesis. Examples of enzymes include glycosyltransferases such as N-acetylglucosaminyltransferases, including N-acetylglucosaminyltransferases I through V and β-1,3-galactosyl-O-glycosyl-glycoprotein β 1,6-N-acetylgucosaminyl transferase (core 2 GlcNAc); fucosyltransferase; N-acetyl galactosaminyltransferases; galactosyltransferases; mannosyltransferases; and glucuronosyltransferases, preferably N-acetylglucosaminyltransferases. Table 1 provides examples of eukaryotic carbohydrate processing enzymes, and their sugar nucleotide donors and acceptors. Table 2 provides a list of prokaryotic carbohydrate processing enzymes.

A nucleoside monomer unit used in the molecules of the present invention is composed of a heterocyclic amine base in β-N-glycosidic linkage with a sugar. Generally, the sugar is ribose, or deoxyribose, and the heterocyclic amine base corresponds to the heterocyclic amine base of the sugar nucleotide donor for a selected carbohydrate processing enzyme. For example, uracil can be selected

for N-acetylglucosaminyltransferases and galactosyltransferases; cytosine for asialo transferases, and guanine for fucosyltransferases.

Structural analogues of the heterocyclic amine bases may also be used. For example, when the base is uracil it may have groups at the C-5 position including but not limited to alkyl or aryl with electron donating and electron withdrawing groups. Hydroxyl groups in the base may also be protected. The sugar may be modified for example, the 2' and 3' hydroxyls may be blocked with acetonide, acylated, or alkylated or substituted with other groups such as halogen.

Specific examples of nucleoside monomer units include uridine, 2'-deoxyuridine, and 5'-amino-5'-deoxy-2',3'-*O*-isopropylidine uridine (for galactosyltransferases and GlcNAc transferases, cytidine, 2'-deoxycytidine, 5'-amino-5'-deoxy-2',3'-*O*-isopropylidinecytidine (for sialo transferases), and guanosine, 2'-deoxyguanosine, 5'-amino-5'-deoxy-2',3'-*O*-isopropylidineguanosine (for fucosyl transferases), respectively.

A nucleoside monomer unit is linked to a spacer monomer unit by coupling appropriate reactive groups such as carboxylic acids, or activated esters thereof (e.g. hydroxybenzotriazole, pentafluorophenol or N-hydroxysuccinimide esters), carboxylic anhydrides (mixed or symmetric), acyl halides, chloroformates, halides, ketones, aldehydes, sulfonyl chlorides, isocyanates, or isothiocyanates, to other reactive functional groups such as amines to form a stable linkage such as an amide, carbamate, amino, sulfonamide, urea, or isourea, preferably an amide linkage. Each of the monomer units may have one or more identical or different reactive groups.

A spacer monomer unit for use in the invention may comprise any functional group that mimics the phosphate/sugar linkage in a sugar nucleotide donor for a carbohydrate processing enzyme, or which interacts with the enzyme by other mechanisms. The spacer monomer unit may have a charged center. Examples of spacer monomer units that can be used in the molecules of the invention include one or more amino acids, preferably a single amino acid, a dipeptide, or tripeptide, or peptidomimetics/ peptide analog.

Amino acids used in the spacer monomer unit may be naturally-occurring or synthetic amino acids, and they can be aliphatic, or aromatic. An amino acid in the spacer monomer unit may be a chiral or achiral amino acid including but not limited to an L-amino acid, a D-amino acid, an α-amino acid, a β-amino acid, or an analog of an amino acid. In addition, one or more amino acids in the spacer monomer unit may be substituted with a substituent group such as an amide, alkyl, amine, halogen, ether, heterocycle, or an acidic group such as –COOH, or SO_3H. The amino acids may be capped with suitable protecting groups as described herein. The amino acid or peptides may comprise acidic amino acid residues including aspartic acid or glutamic acid, and aspartic acid and glutamic acid mono-benzyl esters or t-butyl esters (for example, at the α- and β- positions for the former, and α- and γ- for the latter).

Examples of amino acids that may be used in the spacer monomer unit include L-aspartic acid-α-benzyl ester, L-glutamic acid-γ-benzyl ester, D-aspartic acid-β-benzyl ester, L-aspartic acid-β-benzyl ester, L-glutamic acid-α-benzyl ester, L-tryptophan, 6-aminohexanoic acid, L-valine, m-tosyl-L-histidine, L-leucine, p-methoxy-benzyl-L-cysteine, sarcosine, L-isoleucine, L-asparagine, ω-p-tosyl

L-arginine, ω-nitro-L-arginine, N-ε-CBz-L-lysine, L-glutamine, L-alanine, O-benzyl-L-threonine, O-benzyl-L-tyrosine, L-methionine, O-benzyl-L-serine, L-proline, L-phenylalanine, β-alanine, α-aminoisobutyric acid, homoarginine, homoproline, homoserine, norarginine, norleucine, ornthine, and p-nitrophenylalanine.

Peptidomimetics or peptide analogs may also be used in the spacer monomer unit. Generally, peptidomimetics are structurally similar to a paradigm, such as naturally-occuring peptides, but have one or more peptide linkages optionally replaced for example, by a linkage selected from the group consisting of: $-CH_2NH-$, $-CH_2S-$, CH_2CH_2- , -CH=CH- (cis and trans), $-COCH_2$ -, $-CH(OH)\ CH_2-$, and $-CH_2SO-$ by methods known in the art and further described in the following references: Spatola, A. F. in CHEMISTRY AND BIOCHEMISTRY OF AMINO ACIDS, PEPTIDES, AND PROTEINS, B. Weinstein, eds., Marcel Dekker, New York, p.267 (1983); Spatola, A. F., Vega Data (March 1983), Vol. 1, Issue 3, PEPTIDE BACKBONE MODIFICATIONS (general review); Morley, Trends Pharm Sci (1980) pp. 463-468 (general review); Gaute (1994) Angew. Chem., Int. Ed. Engl., 33:1699-1720; Giannis and Kolter (1993), Angew. Chem., Int. Ed. Engl., 32: 1244-1267; Hudson, D. et al., (1979) Int J Pept Prot Res 14:177-185 (--CH_2NH--, CH_2CH_2--); Spatola et al., (1986) Life Sci 38:1243-1249 (--CH_2--S); Hann (1982) J. Chem. Soc. Perkin Trans. I 307-314 (-CH=CH-, cis and trans); Almquist et al., (1980) J Med Chem 23:1392-1398 (-$COCH_2$-); Jennings-White et al., (1982) Tetrahedron Lett 23:2533 (-$COCH_2$-); Szelke et al., (1982) European Appln. EP 45665 CA: 97:39405 (1982) (-CH(OH) CH_2-); Holladay et al., (1983) Tetrahedron Lett 24:4401-4404 (-$C(OH)CH_2$-); and Hruby (1982) Life Sci 31:189-199 (-CH_2-S-); each of which is incorporated herein by reference. Peptidomimetics or peptide analogs also include peptides wherein the N-terminus is derivatized for example to a $-NXX_1$ group, to a --NXC(O)X group, to a --NXC(O)OX group, to a $--NXS(O)_2X$ group, to a --NHC(O)NHX group where X and X_1 are hydrogen or lower alkyl with the proviso that X and X_1 are not both hydrogen, to a succinimide group, to a benzyloxycarbonyl-NH--(CBZ--NH--) group, to a benzyloxycarbonyl-NH--group having from 1 to 3 substituents on the phenyl ring selected from the group consisting of lower alkyl, lower alkoxy, chloro, and bromo, peptides wherein the C terminus is derivatized to $--C(O)X_2$ where X_2 is selected from the group consisting of lower alkoxy, and $--NX_3X_4$ where X_3 and X_4 are independently selected from the group consisting of hydrogen and lower alkyl.

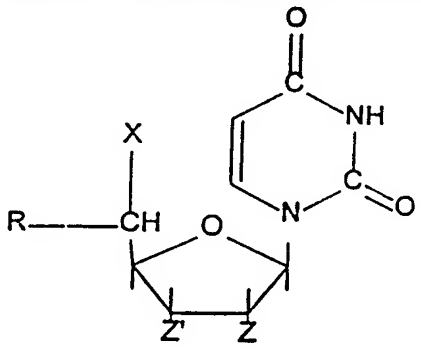
Free functional groups on a nucleoside peptide molecule, in particular free amine groups in the spacer monomer, may be capped using the same or different chemical reactive groups used for a spacer monomer unit coupling to nucleoside monomer units. Examples of cap monomer units include ureas, thioureas, carbamates, and amide residues, which may be part of aromatic rings, non-aromatic rings, heterocyclics, carbocyclics, or fused ring systems. Reactive derivatives of naturally occurring alkaloids such as swainsonine or castanospermine may also be used as cap monomers. Examples of commercially available reagents that may be used to form the cap monomer units include: benzoyl chloride, benzenesulfonyl isocyanate, 4-toluenesulfonyl isocyanate, 2-furonyl chloride, (R)-α-methylbenzyl isocyanate, 4-(trifluoromethylthio)phenyl isocyanate, 2-methoxycarbonyl, phenyl isocyanate, 4-morpholine carbonyl chloride, 1-isothiocyanato-4-(trans-4-octylcyclohexyl) benezene, 3-

(trifluoromethyl)phenyl isocyanate, 1-adamantanecarbonyl chloride, 4-chlorobenezene sulfonyl isocyanate, quinoxay chloride, 2-thiophenecarbonyl chloride, 2-naphthyl isocyanate, 2-thiopheneacetyl chloride, 1-adamantyl isocyanate, 3-cyclopentylpropionyl chloride, pyrolidine carbonyl chloride, 4-trifluoromethoxy-benzoyl chloride, 3-methoxy benzoyl chloride, 4-[4-isothiocyanato phenyl azo] N,N-dimethyl aniline, chloro acetic anhydride, 4-fluoro benzoyl isocyanate, picolinic acid, nicotinic acid, isonicotinic acid, 6-methylnicotinic acid, 3-pydidylacetic acid, trans-3-(3-pyridyl)acrylic acid, (4-pyridylthio)acetic acid, 2-chloronicotinic acid, 6-chloronicotinic acid, 5,6-dichloronicotine acid, 6-hydroxypicolinic acid, 6-hydroxynicotinic acid, 3-hydroxypicolinic acid, 5-chloro-6-hydroxynicotinic acid, 4-pyridoxic, citrazinic acid, 2-furoic acid, 3-furoic acid, 5-bromo-2-furoic acid, 2-thiophenecarboxylic acid, 3-thiophenecarboxylic acid, 4-nitro-3-pyrazolecarboxylic acid, 5-nitro-3-pyrazolecarboxylic acid, 4-hydroxy-7-fluoromethyl-3-quinolinecarboxylic acid, and 4,8-dihydroxyquinoline-2-carboxylic acid. Examples of cap monomers that can be used to cap a free NH_2 and form part of the nucleoside peptide molecules include but are not limited to methyl (Me), formyl (CHO), ethyl (Et), acetyl (Ac), t-butyl (t-bu), anisyl, trifluoroacetyl (Tfa), benzoyl (Bz), 4-methylbenzyl (Meb), thioanizyl, thiocresyl, benzyloxymethyl, 4-nitrophenyl (Pnp), benzyloxycarbonyl (Z), 2-nitrobenzoyl (NBz), 2-nitrophenylsulphenyl (Nps), 4-toluenesulphonyl (Tosyl, Tos), pentafluorophenyl (Pfp), diphenylmethyl (Dpm), 2-chlorobenzyloxycarbonyl (Cl-Z), 2,4,5-trichlorophenyl, 2-bromobenzyloxycarbonyl (Br-Z), triphenylmethyl (Trityl, Trt), 2,2,5,7,8-pentamethyl-chroman-6-sulphonyl (Pmc), t-butyloxycarbonyl (Boc), benzyl (Bzl), benzyloxymethyl (Bom), and 9-fluorenylmethyloxycarbonyl(Fmoc).

Sugar transition state analogues (e.g. GlcNAc analogues) may be coupled to the nucleoside peptide molecules in a position in space close to where a sugar-phosphate bond would be cleaved in a corresponding sugar nucleotide donor.

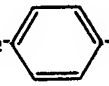
Specific examples of nucleoside peptide molecules of the invention have the formula I:

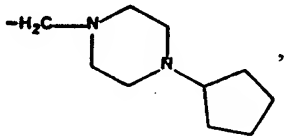
I



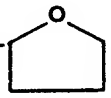
wherein X is H, –COOH, $-OSO_3H$, $(CH_2)qSO_3H$ where q is 0 or 1, or $-OPO_3H$, and R represents $(Y)_m$ where Y is a substituted amide group (e.g. an amide linked amino acid residue) and m is 1-3, Z' and Z are the same or different and represent hydroxyl or alkoxy, or Z' and Z together form an acetonide group, and wherein free NH_2 groups in the compound of the formula I are preferably capped with the cap monomers mentioned herein, preferably with Fmoc or Boc.

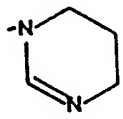
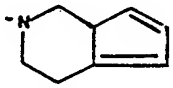
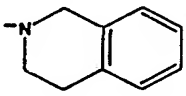
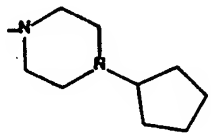
Specific examples of nucleoside peptide molecules of the invention have the formula I wherein X is H, –COOH, $-OSO_3H$, or $(CH_2)qSO_3H$ where q is 0 or 1, Z and Z' are both hydroxyl or together form an acetonide group, R represents $-NHCOR^1$, wherein R^1 represents

(a) $-C(CH_3)(NH_2)CH_2-$  $-R^2$, wherein R^2 is alkoxy; or

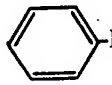
(b) $-CHR^3R^4$ wherein R^3 is hydrogen or $-NH_2$, and R^4 is  $-R^5$ wherein R^5 is halogen, alkyl, or alkoxy,  , $-CH_2N(CH_3)CH_2CH_2R^6$ or $-N(CH_3)CH_2CH_2R^6$

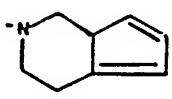
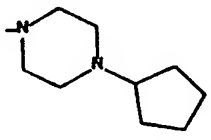
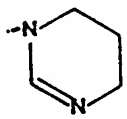
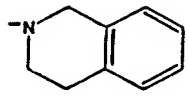
wherein R^6 is halogen,

$-CH_2N(CH_3)CO-$  , $-CH_2N(C_2H_5)CH_2CH(CH_3)OH$, or $-CH_2NHCOCH(CH_3)_2$, or

R^4 represents $(CH_2)_nR^8$ wherein n = 0 to 5, R^8 is halogen,  $-R^9$ wherein R^9 is alkoxy,  ,  ,  ,  ,

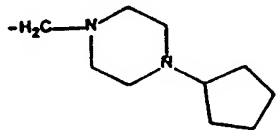
$-N(CH_3)CH_2CH_2R^{10}$ wherein R^{10} is halogen, $-N(C_2H_5)CH_2CH(CH_3)OH$, or $-NHCOCH(CH_3)_2$ and wherein free amino groups are protected with a cap monomer.

In an embodiment of a compound of the formula I of the invention, X is –COOH, and R represents $-NHCOR^1$ wherein R^1 represents $-CHR^3R^4$ wherein R^3 is hydrogen, and R^4 is $(CH_2)_nR^8$ wherein n = 0 to 5, preferably 1 to 4, R^8 is halogen,  $-R^9$ wherein R^9 is alkoxy, halogen, or alkyl,

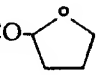
 ,  ,  ,  ,

or $-N(CH_3)CH_2CH_2R^{10}$ wherein R^{10} is halogen, $-N(C_2H_5)CH_2CH(CH_3)OH$, or $-NHCOCH(CH_3)_2$.

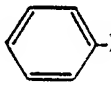
In another embodiment of the invention, a compound of the formula I is provided wherein , X is –COOH, and R represents $-NHCOR^1$ wherein R^1 represents $-CHR^3R^4$ wherein R^3 represents $-NH_2$, and R^4

is  $-R^5$ wherein R^5 is halogen, alkyl or alkoxy, 

$-CH_2N(CH_3)CH_2CH_2R^6$ wherein R^6 is halogen, $-CH_2N(C_2H_5)CH_2CH(CH_3)OH$, $CH_2NHCOCH(CH_3)_2$

or $-CH_2N(CH_3)CO$- 

In a further embodiment of the invention, a compound of the formula I is provided wherein X is $-OSO_3H$, or $(CH_2)qSO_3H$ where q is 0 or 1, R represents $-NHCOR^1$ wherein R^1 represents $-CHR^3R^4$ wherein R^3 represents $-NH_2$, and R^4 is

 -R^5 wherein R^5 is halogen, alkyl, or alkoxy, $-CH_2N(C_2H_5)CH_2CH(CH_3)OH$, or

$-CH_2NHCOCH(CH_3)_2$.

In a particular embodiment, a nucleoside peptide molecule of the formula I is provided wherein X

is $-COOH$, R is represents $-NHCOR^1$ wherein R^1 represents $-C(CH_3)(NH_2)CH_2$  R^2 wherein R^2 is alkoxy.

As used herein the term "alkyl", alone or in combination, refers to a branched or linear hydrocarbon radical, typically containing from 1 through 10 carbon atoms, preferably 1 through 5. Typical alkyl groups include but are not limited to methyl, ethyl, 1-propyl, 2-propyl, 1-butyl, 2-butyl, tert-butyl, or pentyl, preferably methyl or ethyl.

The term "alkoxy" refers to an alkyl linked to the parent molecular moiety through an oxygen atom. Examples of alkoxy groups include O-methyl i.e. methoxy, O-allyl i.e. allyloxy, O-propyl i.e. propoxy, O-butyl i.e. butoxy, and the like, preferably methoxy or allyloxy.

The term "halo" or "halogen", alone or in combination, refers to a member of the family fluorine, chlorine, bromine, or iodine.

Specific examples of nucleoside peptide molecules are shown in Tables 3, 4, and 5.

In the nucleoside peptide molecules of the invention the stereochemistry of chiral carbon atoms in the nucleoside monomer unit, spacer monomer unit, or cap monomer unit can independently be in the R or S configuration, or a mixture of the two. For example, amino acids of the spacer monomer can be in the L-or D-configuration, resulting in the same amino acid, varying only in its stereochemistry. Therefore, the present invention encompasses a nucleoside peptide molecule of the invention as a mixture of diastereomers, as well as in the form of an individual diastereomer, and the present invention encompasses a nucleoside peptide molecule as a mixture of enantiomers, as well as in the form of an individual enantiomer. All optical isomers and racemic forms thereof of the nucleoside peptide molecules of the invention are contemplated herein, and the nucleoside peptide molecules shown herein are intended to encompass all possible optical isomers of the compounds so depicted.

The formation of diastereomers may be carried out pre or post spacer attachment to the nucleoside monomer unit by using L and/or D amino acids during synthesis or by racemizing chiral centers after spacer attachment or construction with base.

Nucleoside peptide molecules of the invention may be present as pharmaceutically acceptable salts. The term "pharmaceutically acceptable salts" encompasses those salts that form by standard acid-base reactions with basic groups and organic or inorganic acids, or acidic groups and bases. Examples of acids include hydrochloric, sulfuric, phosphoric, acetic, succinic, citric, lactic, maleic, fumaric, palmitic, cholic, pamoic, mucic, D-glutamic, d-camphoric, glutaric, phthalic, tartaric, lauric, stearic, salicyclic, methanesulfonic, benezenesulfonic, sorbic, benzoic, cinnamic, and like acids. Examples of bases include LiOH, NaOH, KOK and $Ca(CH_2)$. Basic amino acids (e.g. glycine, ornithine, histidine, phenylglycine, lysine, and arginine) in a spacer monomer unit may be in protonated forms.

Preparation of a Combinatorial Library

A monomer unit is chemically conjugated i.e. covalently linked or coupled, to an adjoining monomer unit or building block to prepare a combinatorial library of the invention. After conjugation, a monomer unit is altered, for example, upon reaction to form a covalent bond, the monomer can lose a water molecule, or can undergo formation of a urea or carbamate group. There are innumerable variations in the nature of the monomer units and in the types of chemical reactions that can be used to chemically conjugate the monomers. In addition, solid phase and solution phase chemistries may be used to synthesize a combinatorial library of the invention.

The building blocks or monomers used in the compounds contained in the library of the invention may be assembled "backwards" i.e. the last building block added to the "growing chain" may be analogous to the 5' terminal end of a peptide or polypeptide. For example, in a library schematically depicted as uridine-spacer-cap, the uridine building block or monomeric unit may be chemically conjugated to an adjoining spacer unit last in time. In this scheme, the cap monomeric unit is generally attached to a solid phase matrix until release of uridine-spacer-cap following the last chemical conjugation reaction.

Examples of processes for preparing compounds in combinatorial libraries of the invention are set out below.

A combinatorial library of the invention where the reactive group on the nucleoside monomer unit is an amine may be prepared using an acetonide, or other suitable protecting groups to temporarily protect chemically active sites. In particular, a library based on uridine structures may be produced using a 5'-deoxy-5'-amino-2',3'-*O*-isopropylidinyl uridine template. The template may be prepared by acetonide blockage of the 2'- and 3'-hydroxyl groups, activation of the 5'hydroxy using mesylation, tosylation, or triflation, subsequent reaction with sodium azide, and reduction (for example, see Figures 1 to 4). A spacer monomer unit comprising an amino acid, dipeptide, or tripeptide which is suitably protected, e.g. N-t-butyloxycarbonyl (Boc), or N-9-fluorenylmethyloxycarbonyl(Fmoc)-protected, may be coupled with the uridine template in free base form ((for example, see Figure 5). This is followed by deprotection (see for example, Figure 6). Purification of the nucleoside peptide monomer unit and spacer unit is carried out using conventional methods, and the free amine bases may be capped with for

example, carboxylic acids, anhydrides, esters, isocyanates, isothiocyanate, acid chloride, or aldehydes (see for example, Figure 7).

The invention also contemplates intermediates used in the processes of the invention, including nucleoside peptide molecules of the combinatorial libraries of the invention having a protected hydroxy in the heterocyclic amine base and/or which may be blocked at the 2' or 3' hydroxyls.

The spacer monomer unit may be coupled to the nucleoside monomer unit using a synthetic strategy that consists of three chemical steps and two purification steps, all of which can be automated. Examples of reaction schemes for adding the spacer monomer unit are shown in Figures 5 and 6. The reactions may be performed in deep well (1 or 2 mL), 96-well-format microtitre plates. The first step is to couple Boc- protected amino acids, dipeptides or tripeptides to the uridine template in free base form. This is followed by concomitant N-deprotection and acetonide deblockage using an excess of TFA. The TFA salts are neutralized by ion exchange slurrying using a Polyfiltronics™ unifilter plate (in 96 well format), allowing for the generation of free amine residues. The free amine groups are capped for example with isocyanates, isothiocyanates, carboxylic acids, sulfonyl chlorides, and acyl chlorides to give five libraries of products. Purification, if necessary, can be carried out by slurrying with aminomethyl resin, which scavenges any excess capping reagent, or with alumina silica or Florisil™ which retains excess reagents and byproducts. The slurrying can be performed in a Polyfiltronics™ plate. The free terminal amine groups may also be capped with aldehydes under reductive amination conditions. For benzyl ester-protected derivatives (e.g. aspartic acid and glutamic acid residues in the spacer monomer unit), manual transfer hydrogenation using ammonium formate, Pd-C (10%, wet) and methanol, hydrolysis (TFA, H_2O) or saponification (methanol, KOH, H_2O) can be performed to liberate the carboxylate and racemize chiral amino acid fragments if desired.

An alternative reaction scheme for adding spacer monomer units using an Fmoc strategy is shown in Figure 7. The reactions may be performed in deep well (1 or 2mL), 96-well format microtitre plates if desired. The first step is to couple Fmoc-protected amino acids, dipeptides or tripeptides to the free amine group of the uridine monomer template. This is followed by Fmoc deprotection with morpholine in DMF as solvent. This method liberates the free terminal amine without removing the isopropylidene protecting group. No neutralization step is necessary and morpholine is easily removed by evaporation under reduced pressure. The terminal amines are then capped as required, as described above. The acetonide protecting group can be removed from all or selected capped or uncapped uridine peptides in a final reaction sequence by treatment with TFA at room temperature followed by evaporation of reagents and solvent under reduced pressure (see Figure 8).

A combinatorial library of the invention where an amide group links a nucleoside monomer unit and a spacer monomer unit may be prepared by forming a compound of the formula I where R represents $-NHCOCHR^3R^4$ wherein R^3 is NH_2 using the method as described in N. P. Damodaran et al. J. Am. Chem.Soc. 93, 3812, 1971. The free amino form of the compound is subjected to condensation with a corresponding ester of R^3 in aqueous DMF in the presence of N-methyl morpholine at an appropriate temperature. Other reactive esters such as N-hydroxy succinimidyl, hydroxybenzotriazole,

or pentafluorophenyl esters, or other reactive esters commonly used in peptide synthesis may also be used. For example, synthesis of a compound of the formula I where R^4 is $(CH_2)_nR^8$ where n is 2 and R^8 is halogen (compound **A** in Table 3) can be achieved by using the methyl ester of 1-fluorobutyric acid. Similarly, compounds **B**, **C**, and **D** in Table 3 can be synthesized by using an ester of the corresponding acid, which can be synthesized by conventional methods. Compounds **E** to **J** in Table 3 can be synthesized by using the appropriate esters of the corresponding acids, which are commercially available. For compounds **F** to **H** in Table 3, prior to condensation, the free amino group in the esterified reagent is blocked with a suitable group.

A combinatorial library containing the selected compounds shown in Tables 4 and 5 where X is sulphate can be synthesized by a similar condensation of an ester with a free amine.

Predetermined compounds in the combinatorial library where C-5 of a uridine has different alkyl and aryl groups may be prepared by mercuration of commercially available UDP with mercuric acetate to give UDP-C-5-mercuric acetate, which on treatment with an appropriate alkene compound in the presence of potassium tetrachloropalladate produces the corresponding C-5-alkene derivative. On selective reduction, these compounds give C-5-alkyl compounds. This type of derivatization is known as the Heck reaction and it can be carried out in a variety of ways known in the art (Ryabov, Synthesis (1985) 233-252; and Heck, Org. React. (1982) 27: 345-390).

A transition state analogue of a sugar which is transferred by a sugar nucleotide donor may be coupled to a nucleoside peptide molecule of the invention. For example, a GlcNAc cation analogue can be generated, and prepared in a form that would allow it to be coupled to a uridine ribose molecule of the invention.

Bioassays

The combinatorial library of the invention contains putative inhibitors of carbohydrate processing enzymes. Inhibitors with appropriate selectivity and activity against a particular carbohydrate processing enzyme may be selected using conventional bioassays and the bioassays described herein. Bioassays may be adapted for high throughput screening incorporating automation and robotics to enable testing thousands to millions of compounds in a relatively short time. Preliminary screening of 5408 compounds from a library of the invention, revealed that 2-3% of the compounds had inhibitory activity in conventional core 2 GlcNAc-T, GlcNAc-TV, and GlcNAc-TI assays.

Once "lead" compounds are identified using the screening techniques, combinatorial chemistry methods can be used to optimize the initial leads. The optimized analogs/variants can be tested in the same screening assays that identified the initial lead.

The methods designed by the present inventors described herein use simple, and rapid functional assays that can identify one or more active ingredients in tested pools without the need for a long deconvolution process. The assays are used in robotics systems that can handle large numbers of samples for proportioning, mixing, and sample-handling. The invention therefore makes available robotics that can perform multiple chemical reactions at variable temperatures, and subsequently handle work up and characterization of bioactive leads. The selection means enable identification of

active compounds within the combinatorial libraries that can generate affinity enrichment or affinity selection, and this enrichment and selection may be followed by mass spectroscopic identification of any bioactive compounds.

The present invention contemplates a solid-phase bioassay for identifying a compound in a combinatorial library of the invention having inhibitory activity against a carbohydrate processing enzyme including glycosyltransferases or glycosidases. The method is particularly useful for drug screening. The solid-phase bioassay involves coupling a carbohydrate acceptor for the carbohydrate processing enzyme to a polymer and coating onto a carrier or support. A carbohydrate processing enzyme, a sugar nucleotide donor labeled with a detectable substance, and a test compound are added, and the detectable change produced by the detectable substance is measured.

Examples of polymers to which an acceptor may be coupled include polyacrylamide. The carrier or support may be for example nitrocellulose, or glass, gabbros, or magnetite. The support material may have any possible configuration including spherical (e.g. bead), cylindrical (e.g. inside surface of a test tube or well, or the external surface of a rod), or flat (e.g. sheet, test strip).

Examples of detectable substances include, but are not limited to, radioisotopes (e.g., ^{3}H, ^{14}C, ^{35}S, ^{125}I, ^{131}I), fluorescent labels (e.g., FITC, rhodamine, lanthanide phosphors), luminescent labels such as luminol, enzymatic labels (e.g., horseradish peroxidase, beta.-galactosidase, luciferase, alkaline phosphatase, acetylcholinesterase), and biotinyl groups (which can be detected by marked avidin e.g., streptavidin containing a fluorescent marker or enzymatic activity that can be detected by optical or calorimetric methods). In an embodiment of the invention, the detectable substance is a radioactive material, most preferably tritium.

A carbohydrate processing enzyme used in the method may be obtained using conventional extraction methods from natural sources, it may be a recombinant enzyme, or it may be obtained from commercial sources.

In an embodiment of the invention, the assay involves coupling carbohydrate acceptors to a polymer (e.g. polyacrylamide) and coating onto a carrier, such as the surface of 96 well plastic plates. The glycosyltransferase reaction is performed with recombinant enzymes and a tritiated sugar-nucleotide donor, followed by washing, addition of scintillation counting fluid, and measurement of radioactivity with a ß-counter. Glycopolymer construction and coating of the plastic plates, enzyme and substrate concentrations, and linearity with time were optimized using UDP-GlcNAc:Galß1-3GalNAc-R ß1-6-N-acetylglucosaminyltransferase (GlcNAc to GalNAc) (i.e. core 2 GlcNAc-T), a rate-limiting reaction for expression of polylactosamine and the selectin ligand sialy Lewis^{x}. Polylactosamine expression has been associated with malignant transformation (Itzkowitz, SH *et al.*, *Cancer Res.*, 46, 2627-2632, 1986; Kim YS et al., Cancer Res. 46, 5985-5992, 1986,), development (Pennington JE *et al.*, *J.Embryol.*, 90, 335-361, 1985) and proliferative activation of lymphocytes (Higgins EA *et al.*, *J.Biol.Chem.*, 266, 6280-6290, 1991). Polylactosamine structures have been shown to play a significant role in cell-cell and cell-substratum adhesion processes (Zhu BC and Laine RA, *J.Biol. Chem.*, 260, 4041-4045, 1985; Laferte' S and Dennis JW, *Cancer Res.*, 48, 4743-4748, 1988). Additionally, they

may act as ligands for mammalian lectins (Merkle RK and Cummings RD, *J.Biol.Chem.*, 263, 16143-16149, 1988).

In a screen to detect core 2 GlcNAc-T inhibitors in a microbial extract library, the CV for positive controls was +/- 9.4 %, and complete concordance for hit validation was observed between the solid phase assay and a standard solution assay.

A glycosyltransferase assay can be used to identify inhibitors of a variety of carbohydrate processing enzymes, including the enzymes described herein, preferably core 2 GlcNAc-T, GlcNAc-TI and GlcNAc-TV.

Lectin-sensitivity assays have been largely employed to study the carbohydrate patterns of cell lines. By specifically binding to oligosaccharide structures at the cell surface, lectins generally exert a cytotoxic effect causing growth disadvantage. L-PHA is a lectin which recognizes tri- and tetra-antennary N-linked oligosaccharides carrying the structure (Galβ1,4GlcNAcβ1,6)Galβ1,4-GlcNAcβ1,2Manα), thus representing a valid probe for detection of β 1,6 branched, complex-type oligosaccharides. These structures are associated with tumor progression and appear on malignant cells (Dennis et al., 1986) [for example the murine lymphoreticular, highly metastatic, tumor model MDAY-D2 line (VanderElst and Dennis, 1991)]. Reduction and/or truncation of cell surface N-linked carbohydrate chains in MDAY-D2 cells is directly correlated with decreasing levels of L-PHA sensitivity and, in turn, with improving cell proliferation even in the presence of the lectin. Thus, such a system can be exploited for revealing any means blocking the biosynthesis of 1,6, branched N-linked structures. Given the functional significance of complex N-oligosaccharides during malignant transformation, large-scale L-PHA assays have been developed by the present inventors to identify compounds in the combinatorial libraries that are new inhibitors of the N-linked oligosaccharide processing pathway.

The terms "N-linked oligosaccharide processing" or "N-linked oligosaccharide processing pathway" refer to the biosynthetic pathway for the *in vivo* synthesis of glycoproteins with N-linked oligosaccharides. N-linked oligosaccharides are linked to the amide N in the sidechain of Asn in the consensus sequence Asn-X-Ser/Thr of the protein moiety, where X can be any amino acid. The method of the invention can be particularly applied to identify compounds that inhibit complex-type N-linked oligosaccharides, in particular β 1,6- branched complex-type oligosaccharides associated with tumor growth and metastasis. N-linked oligosaccharide processing involves the synthesis of a precursor molecule, transfer of the precursor to Asn by oligosaccharyltransferase followed by further processing by membrane-bound glucosidases and endoplasmic reticulum α 1,2-mannosidase, and transport from the rough endoplasmic reticulum to the Golgi stacks. In the Golgi stacks, further processing occurs depending on the final destination of the glycoprotein and it may involve lysosomal enzymes or nonlysosomal enzymes. Complex and hybrid type oligosaccharide chains are synthesized through the second nonlysosomal processing pathway and residues can be added by enzymes including Golgi mannosidase I (α1,2 specific), and N-acetylglucosaminyltransferases I, II, and III (A description of the N-linked processing pathway may be found at http://www.uni.mainz.de/~frosc000/STRU22.html).

The L-PHA method of the invention can be used to identify compounds that inhibit all steps in the N-linked oligosaccharide pathway prior to β1,4 Gal transferase, including compounds that inhibit the carbohydrate processing enzymes described herein, and Golgi α-mannosidases.

In an embodiment of the invention, a fully automated enzymatic method is contemplated that is based on measurement of alkaline phosphatase activity. The method is based on the observation that the number of cells and their level of alkaline phosphatase activity are closely correlated. The method employs a colorimetric assay to monitor cell proliferation of transformed cells after L-PHA treatment. The reaction mixture is directly added to cells growing in their own medium. Thus, the method can be carried out in a single step, without removal of the culture medium or cell pelletting and washing, thereby permitting the fully automated procedures. The assay method is also highly reproducible (CV=4%) and inexpensive, thus representing a valuable tool when large-scale experiments are performed. The reaction is linear with time in a wide time interval (5-180 min), and the Km value of the enzyme for the substrate para-nitrophenylphosphate is relatively low (0.81 mM). Incubation time and substrate concentration can be changed in order to modulate the velocity of the reaction and adjust the protocol, for automation and timing purposes, to the number of samples. Use of a robotic platform also allows simultaneous processing of large numbers of samples, e.g. thirty-six 96-well plates.

Therefore, an automated method is provided for testing a compound for its ability to inhibit N-linked oligosaccharide processing comprising (a) incubating the compound with cells expressing N-linked oligosaccharides (preferably β 1,6 branched, complex-type oligosaccharides) in the presence of L-PHA, and measuring alkaline phosphatase activity; and (b) comparing to a control in the absence of the compound wherein higher alkaline phosphatase activity indicates that the compound has the ability to inhibit N-linked oligosaccharide processing. The method may be used to identify compounds which inhibit all steps in the N-linked oligosaccharide pathway prior to β1,-4 Gal-transferases, including compounds that inhibit the carbohydrate processing enzymes described herein, in particular N-acetylglycosaminyltransferases, including N-acetylglucosaminyltransferases I, II and V. The method may also be used to identify compounds that inhibit Golgi α-mannosidases.

The automated method of the invention can generally be used to identify antagonists of cell growth inhibitors, such as TGF-β, IL-1γ , TNFα, and IFN. Therefore, the invention broadly contemplates a method comprising (a) reacting a test compound with cells expressing N-linked oligosaccharides in the presence of a cell growth inhibitor; (b) measuring alkaline phosphatase activity ; and (c) comparing to a control in the absence of the test compound wherein an increase in alkaline phosphatase activity indicates that the compound has the ability to antagonize the cell growth inhibitor.

Cells which can be used in the methods of the invention include MDAY-D2, L1210, melanoma tumor cells, and human tumor cells such as SW 480, LS174T, HT-29, WiDr, T2, MDA-231, MCF7, BT-20, Hs578T, K562, Hs578T, SK-BR-3, CY 6T, MDA-468, H23, H157, H358, H1334, H1155, H28, H460, Hmesol, H187, H510A, N417, H146, H1092, H82 (Restifo, N. P. et al, J. Exper. Med. 177:265-272, 1993). The cell lines may contain either constitutive or inducible enzyme activity such as osteoblastic cell lines.

Cell proliferation is measured by measuring alkaline phosphatase activity. Alkaline phosphatase may be measured using conventional methods for example by using para-nitrophenylphosphate as a substrate and measuring absorbance at about 405nm.

The conditions for carrying out the method will be selected having regard to the nature of the compound and the cells employed. For example, if the cells are MDAY-D2 tumor cells a concentration of about 1-6 x 10^3 cells, preferably 5 x 10^3 may be used. The MDAY-D2 cells are generally cultured for about 10 to 30 hours, preferably 16 to 20 hours, followed by addition of L-PHA at a concentration of about 50 to 150 μg/mL, preferably 100 μg/mL. The alkaline phosphatase assay mixture may contain a buffer e.g. diethanolamine buffer, and para-nitrophenylphosphate at an initial concentration of about 1.5 to 4 mM, preferably 2 to 3 mM, most preferably 2.5 mM.

Utility of Inhibitors

Small molecule inhibitors with appropriate selectivity and activity against a particular carbohydrate processing enzyme can be selected from the combinatorial libraries of the invention using high throughput screening bioassays. The selected small molecule inhibitors will have valuable pharmacological properties. In particular, the inhibitors will be useful in the treatment and prophylaxis of tumor growth and metastasis of tumors. Anti-metastatic effects of inhibitors can be demonstrated using a lung colonization assay. For example, melanoma cells treated with an inhibitor may be injected into mice and the ability of the melanoma cells to colonize the lungs of the mice may be examined by counting tumor nodules on the lungs after death. Suppression of tumor growth in mice by the inhibitor administered orally or intravenously may be examined by measuring tumor volume.

A small molecule inhibitor can have particular application in the prevention of tumor recurrence after surgery i.e. as an adjuvant therapy.

A small molecule inhibitor can be especially useful in the treatment of various forms of neoplasia such as leukemias, lymphomas, melanomas, adenomas, sarcomas, and carcinomas of solid tissues in patients. In particular, the small molecule inhibitors can be used for treating malignant melanoma, pancreatic cancer, cervico-uterine cancer, ovarian cancer, cancer of the kidney such as metastatic renal cell carcinoma, stomach, lung, rectum, breast, bowel, gastric, liver, thyroid, head and neck cancers such as unresectable head and neck cancers, lymphangitis carcinamatosis, cancers of the cervix, breast, salivary gland, leg, tongue, lip, bile duct, pelvis, mediastinum, urethra, bronchogenic, bladder, esophagus and colon, non-small cell lung cancer, and Kaposi's Sarcoma which is a form of cancer associated with HIV-infected patients with Acquired Immune Deficiency Syndrome (AIDS). The inhibitors may also be used for other anti-proliferative conditions such as bacterial and viral infections, in particular AIDS.

A small molecule inhibitor of the present invention can be used to treat immunocompromised subjects. For example, they can be used in a subject infected with HIV, or other viruses or infectious agents including bacteria, fungi, and parasites, in a subject undergoing bone marrow transplants, and in subjects with chemical or tumor-induced immune suppression.

A small molecule inhibitor can be used as hemorestorative agents and in particular to stimulate bone marrow cell proliferation, in particular following chemotherapy or radiotherapy. The

myeloproliferative activity of an inhibitor of the invention may be determined by injecting the inhibitor into mice, sacrificing the mice, removing bone marrow cells and measuring the ability of the inhibitor to stimulate bone marrow proliferation by directly counting bone marrow cells and by measuring clonogenic progenitor cells in methylcellulose assays. The inhibitors can also be used as chemoprotectants and in particular to protect mucosal epithelium following chemotherapy.

A small molecule inhibitor of the invention also can be used as an antiviral agent in particular on membrane enveloped viruses such as retroviruses, influenza viruses, cytomegaloviruses and herpes viruses. A small molecule inhibitor can also be used to treat bacterial, fungal, and parasitic infections. For example, a small molecule inhibitor can be used to prevent or treat infections caused by the following: *Neisseria* species such as *Neisseria meningitidis*, and *N. gonorrheae; Chlamydia* species such as *Chlamydia pneumoniae, Chlamydia psittaci, Chlamydia trichomatis; Escherichia coli, Haemophilus* species such *as Haemophilus influenza; Yersinia enterocolitica; Salmonella* species such as *S.typhimurium*; *Shigella* species such as *Shigella flexneri*; *Streptococcus* species such as *S.agalactiae* and *S. pneumoniae*; *Bacilllus* species such as *Bacillus subtilis; Branhamella catarrhalis; Borrelia burgdorfer; Pseudomonas aeruginosa; Coxiella burnetti; Campylobacter* species such as *C.hyoilei*; *Helicobacter pylori*; and, *Klebsiella* species such *as Klebsiella pneumoniae.*

A small molecule inhibitor can also be used in the treatment of inflammatory diseases such as rheumatoid arthritis, asthma, inflammatory bowel disease, and atherosclerosis.

A small molecule inhibitor can also be used to augment the anti-cancer effects of agents such as interleukin-2 and poly-IC, to augment natural killer and macrophage tumoricidal activity, induce cytokine synthesis and secretion, enhance expression of LAK and HLA class I specific antigens; activate protein kinase C, stimulate bone marrow cell proliferation including hematopoietic progenitor cell proliferation, and increase engraftment efficiency and colony-forming unit activity, to confer protection against chemotherapy and radiation therapy (e.g. chemoprotective and radioprotective agents), and to accelerate recovery of bone marrow cellularity particularly when used in combination with chemical agents commonly used in the treatment of human diseases including cancer and acquired immune deficiency syndrome (AIDS). For example, a small molecule inhibitor can be used as a chemoprotectant in combination with anti-cancer agents including doxorubicin, 5-fluorouracil, cyclophosphamide, and methotrexate, and in combination with isoniazid or NSAID.

The term "patient" herein refers to a warm-blooded animal such as a mammal which is afflicted with a particular disease state or condition as described herein. Examples of animals within the scope of the meaning of the term are dogs, cats, rats, mice, horses, bovine cattle, sheep, and humans.

Small molecule inhibitors can be converted using customary methods into pharmaceutical compositions. The pharmaceutical compositions contain the inhibitors either alone or together with other active substances. Such pharmaceutical compositions can be for oral, topical, rectal, parenteral, local, inhalant, or intracerebral use. They are therefore in solid or semisolid form, for example pills, tablets, creams, gelatin capsules, capsules, suppositories, soft gelatin capsules, liposomes (see for example, U.S. Patent No. 5,376,452), gels, membranes, and tubelets. For parenteral and intracerebral

uses, those forms for intramuscular or subcutaneous administration can be used, or forms for infusion or intravenous or intracerebral injection can be used, and can therefore be prepared as solutions of the inhibitors or as powders of the inhibitors to be mixed with one or more pharmaceutically acceptable excipients or diluents, suitable for the aforesaid uses and with an osmolarity which is compatible with the physiological fluids. For local use, those preparations in the form of creams or ointments for topical use or in the form of sprays should be considered; for inhalant uses, preparations in the form of sprays should be considered.

The pharmaceutical compositions can be prepared by per se known methods for the preparation of pharmaceutically acceptable compositions which can be administered to patients, and such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985). On this basis, the pharmaceutical compositions include, albeit not exclusively, the inhibitors in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids.

An inhibitor can be indicated as a therapeutic agent either alone or in conjunction with other therapeutic agents or other forms of treatment (e.g. chemotherapy or radiotherapy). An inhibitor can be used to enhance activation of macrophages, T cells, and NK cells in the treatment of cancer and immunosuppressive diseases. By way of example, an inhibitor can be used in combination with anti-proliferative agents, antimicrobial agents, immunostimulatory agents, or anti-inflammatories. In particular, an inhibitor can be used in combination with anti-viral and/or anti-proliferative agents, such as Th1 cytokines including interleukin-2, interleukin-12, and interferon-γ, and nucleoside analogues such as AZT and 3TC. The compounds of the invention may be administered concurrently, separately, or sequentially with other therapeutic agents or therapies.

Compositions containing small molecule inhibitors can be administered for prophylactic and/or therapeutic treatments. In therapeutic applications, compositions are administered to a patient already suffering from a disease or condition as described above, in an amount sufficient to cure or at least alleviate the symptoms of the disease and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose". Amounts effective for this use will depend on the severity of the disease, the weight and general state of the patient, the nature of the administration route, the nature of the formulation, and the time or interval at which it is administered.

In prophylactic applications, compositions containing small molecule inhibitors are administered to a patient susceptible to or otherwise at risk of a particular disease. Such an amount is defined to be a "prophylactically effective dose". In this use, the precise amounts depend on the patient's state of health and weight, the nature of the administration route, the nature of the formulation, and the time or interval at which it is administered.

It will be appreciated that where major amounts are administered for a therapeutic or prophylactic treatment, it may be advisable to divide these into several administrations over the course of a day.

The following examples illustrate the invention.

EXAMPLE 1

Combinatorial Synthesis of GnT-V Inhibitors

Below, the synthesis of 5'-deoxy-5'-amino-2',3'-O-isopropylidinyl uridine and peptide-branched derivatives of 5'-deoxy-5'-amino-2',3'-O-isopropylidinyluridine are described. The invention contemplates intermediates, 2,3-O-isopropylidene-5-O-methanesulfonyl uridine, and 5-deoxy-5-azido-2,3-O-isopropylidenyluridine derivatives.

I. Synthesis of 5'-deoxy-5'-amino-2',3'-*O*-isopropylidenyl uridine

A. Preparation of 2,3-O-isopropylidenyluridine (Figure 1)

Uridine (65.0 g, 266.2 mmoles), camphor sulfonic acid (1.0 g, 4.3 mmoles), 2,2-dimethoxy propane (98.0 mL, 798.5 mmoles), and acetone (anhydrous, 1000 mL) were stirred vigorously for 24 h at room temperature. The reaction was monitored by TLC (solvent system 7:93, MeOH:$CHCl_3$). Once the starting material was consumed, triethylamine (1.12 mL, 8.6 mmol) was added and the mixture stirred for another one hour. The acetone was evaporated under reduced pressure (<40 °C) to give a white powder (77.0 g), which was used for the next step without any purification.

B. Preparation of 2,3-O-Isopropylidene-5-O-methane sulfonyl uridine (Figure 2)

The crude 2,3-O-isopropylidenyl uridine (77.0g; obtained in step A) was dissolved in DMF. Triethylamine (74.2 mL) was added and the mixture was cooled to 0°C. Methanesulfonyl chloride (31.2 mL) was then added dropwise during a 30-60 min. period while rapid stirring continued. After an additional 1 hr of stirring at room temperature, the DMF was evaporated. The residue was dissolved in ethyl acetate (2.0 L) and washed three times with water (3 X 250 mL). The organic layer was dried over $MgSO_4$, filtered, the filter cake rinsed with some EtOAc and the solvent evaporated. The light yellow residue (thick syrup) was used directly for the next step without further purification. The reaction was monitored by TLC (7:3, ethyl acetate : hexane).

C. Preparation of 5- azido-5-deoxy -2,3-O-isopropylidenyl uridine (Figure 3)

The crude mesylate (obtained from step B above) was dissolved in DMF (400 mL, reagent grade) and stirred at 60°C with sodium azide (34.6g, 532 mmol) for 12 hrs until complete consumption of the mesylate was observed by TLC (TLC 7:3, ethyl acetate: hexane). The mixture was filtered through a Celite bed. The filtrate was evaporated to dryness. The residue was dissolved in ethyl acetate (2.0 L) and washed three times with water (3 X 250 mL). The organic layer was dried over $MgSO_4$ and evaporated under reduced pressure at 30°C. The solid residue was recrystallized from ethyl acetate/hexane (1:1) to afford 65.0g of the desired azido uridine as a white crystalline solid (mp 118°C). ^1H NMR (500 MHz, CD_3OD): 1.34, 1.53 (2s, 6H); 3.52 (dd, 1H, J = 4.4, 12.9 Hz); 3.6 (dd, 1H, J = 5.9, 12.9 Hz); 4.2 (ddd, 1H, J = 4.4, 4.4, 6.2 Hz); 4.8 (dd, 1H, J= 4.2, 6.2 Hz); 5.06 (dd, 1H, 2.3, 6.2 Hz); 5.7 (d, 1H, J = 7.8 Hz); 5.78 (d, 1H, J = 2.2 Hz); 7.65 (d, 1H, J = 8.0 Hz).

D. Preparation of 5-amino-5-deoxy -2,3-O-isopropylidenyl uridine (Figure 4)

5.0g of azide was dissolved in ethanol (150-200mL). To this solution, $Pd(OH)_2$ and $NaHCO_3$ was added. The reaction flask was evacuated and filled with H_2 gas. This was repeated three times and the mixture was stirred for 3-6h at room temperature under H_2. The mixture was filtered through a

Celite bed and evaporated. The residue was used for capping and peptide coupling reactions. ^{1}H NMR (500 MHz, $CDCl_3$): 1.35, 1.57 (2s, 6H); 2.95 (dd, 1H, J = 6.0, 13.6 Hz); 3.06 (dd, 1H, J = 4.6, 13.6 Hz); 4.1 (appear as dd, in fact it is ddd, 1H, J = 4.3, 4.35, 6.4 Hz); 4.76 (dd, 1H, J= 4.5, 6.4 Hz); 4.95 (dd, 1H, 2.6, 6.4 Hz); 5.7 (d, 1H, J = 2.4 Hz); 5.73(d, 1H, J = 8.1 Hz); 7.38(d, 1H, J = 8.2 Hz).

II. Coupling Procedure (Figure 5)

Free amine (Figure 5) was dissolved in dichloromethane (250 mL) and WSC.HCl [1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide .HCl, 1.2 eq] was added. To this clear solution, N-t-butoxycarbonyl protected amino acids (1.0 eq) were added and stirred under argon for 1-3 h. The reaction was monitored by TLC (7: 93, MeOH/$CHCl_3$). After the reaction was complete, more dichloromethane was added and the solution washed with water (for a few amino acids like glutamine, ω-nitroarginine, asparagine water wash is not possible, because the derivatives are water soluble), and the organic layer was dried over $MgSO_4$ and evaporated . The residue was purified by column chromatography (eluent 2-5% MeOH in CH_2Cl_2) to give white solids. Yields vary from 75-85%). This procedure was performed on 25.0g scales. In a similar fashion, free amine (1 eq), N-FMOC-protected amino acids (1.1eq) and HBTU in DMF (1.1 eq) were reacted to give N-FMOC protected uridine monopeptides.

III. N-Boc Deprotection (Figure 6)

Uridine peptides (Figure 6) were treated separately with TFA/$CHCl_3$/H_2O (3:4:1) at room temperature for 12 hours. Excess reagent and solvent were evaporated under reduced pressure. The residue was dissolved in methanol, treated with OH^- resin until neutral, filtered, and evaporated to dryness.

IV. Capping (Figure 8)

Fully deprotected uridine monopeptide and dipeptide derivatives were treated separately with capping reagents (acyl chloride, isocyanate, and thioisocyanate, 1.2 eq) and diisopropylethyl amine (1.5 eq) in DMF at room temperature. After 12h, solvent was removed and the residues were dissolved in methanol. These solutions were treated with aminomethylated polystyrene resin for 48 hours to quench excess capping reagent. Mixtures were filtered, evaporated, and dissolved in DMSO.

N-t-Boc and N-FMOC-deprotected dipeptides (1eq) were also capped with various carboxylic acid caps (1.05-1.2 eq) in the presence of HBTU (1.05-1.2 eq) in DMF. The solvent was evaporated under reduced pressure (temp ≤ 60°). The residues were dissolved in 8/8/1 [MeCN/MeOH/H_2O], and then filtered individually through a pad of basic alumina in a 96-well format using polytronics filter plates. The solvents were again evaporated under reduced pressure (≤ 40°), and the residues diluted in DMSO for storage and testing.

Fully deprotected monopeptide and dipeptide derivatives were also successfully capped separately with a variety of carboxylic acids (1.1 equiv.) with ethyl diisopropylamine in DMF via a carbodiimide coupling with or without HOBT or by an HBTU assisted protocol.

EXAMPLE 2

ABBREVIATIONS

Gal	D-galactose

GalNAc	D-N-acetylgalactosamine
GlcNAc	D-N-acetylglucosamine
FCS	fetal calf serum
T	transferase

A solid-phase glycosyltransferase assay was developed for drug screening. Glycosyltransferases catalyze the formation of glycosydic linkages between monosaccharides donated by sugar-nucleotide, and specific oligosaccharide acceptors. The solid-phase assay was illustrated for core 2 GlcNAc-T and can be adapted for other glycosyltransferases. The assay utilizes multivalent oligosaccharide acceptors linked to polymer coated plastic plates and thereby eliminates the need for chromatographic separation of product.

MATERIALS AND METHODS

Chemicals: Poly[N-(acryloyloxy)succinimide] (pNAS) (**1**, Figure 9) with a viscosity-average molecular weight M_v of 42.1 kDa (DP ~250) was prepared according to Mammen et al. (Mammen et al J. Med. Chem., 38, 4179-41901995) . Disaccharide Galβ1-3GalNAcα-$O(CH_2)_3S(CH_2)_2NH_2$ (2) (core 2 GlcNAc-T acceptor) and [GlcNAc(β1-2)]Man(β1-6)Glc(β-$O(CH_2)_3S(CH_2)NH_2$) were prepared from the corresponding allyl glycoside following a procedure described by Roy and Tropper (R. Roy and F.D. Tropper, J. Chem. Soc. Chem. Commun. 1058 (1988); Glycoconjugate J. 5:203 (1988)). Galβ1-3GalNAcα-pNp and GalNAcα-polymers UDP-6-[^{3}H]-N-acetylglucosamine (16.0 Ci/mmol) were purchased from Toronto Research Chemicals (Toronto, Canada) while non labelled UDP-6-N-acetylglucosamine was obtained from Sigma Chemicals.

Glycopolymer syntheses: Poly[N-(acryloyloxy)succinimide] (**1**) was first treated at room temperature with the amine-terminated T-antigen disaccharide **2** in DMSO (16 h) to provide a core copolymer containing one sugar residue for every ten N-substituted acrylamide residues. The active ester-containing polymer was then treated at room temperature for three hours with excess primary amines (ammonia, methylamine, ethylamine, or propylamine) to give four different copolymers **3-6** having the same comonomer ratios but differing by the lipophilicity of the copolymer backbones. The glycopolymers were then purified by size exclusion chromatography over BioGel P-10 using water as eluent. Alternatively, disaccharide **2** was treated with methacryloyl chloride and the resulting monomer was copolymerized with methacrylamide to provide copolymer **8**, while direct copolymerization of allyl glycoside precursor of **2** with acrylamide gave copolymer **10**. Using the same strategy, copolymer acceptors **11-14** for GlcNAc-T V were prepared using the same core pNAS 1 and molar ratios of acrylamide:sugar of 10:1 (Figure 10).

Recombinant core 2 GlcNAc-T: A truncated form of core 2 GlcNAc-T cDNA, lacking 37 amino acids from the N-terminus was prepared by PCR. The truncated cDNA was cloned in-frame into pPROTA vector (Sanchez-Lopez et al., J. Biol. Chem. 263, 11892-118991988) for expression as a secreted protein A chimeric protein. The expression vector was co-transfected into CHO cells, along with pSV2neo, in a 10:1 molar ratio, using a calcium phosphate method. Cells were cultured in the presence of 800 μg/mL of G418, and resistant cell clones were selected, and tested for core 2 GlcNAc-T activity in culture medium. The representative clone 614 C2 showed stable expression of core 2

GlcNAc-T activity, and was selected for enzyme production. The cells were routinely propagated in MEM medium containing 5% Fetal Bovine Serum and G418 (0.2 mg/mL). IgG-Sepharose Fast Flow™ beads (Pharmacia Biotech) were added in a ratio of 5 µl of a 50% bead slurry, 2.5 µl of 2 M Tris•HCl pH 8.0, and 5 µl of 10% Tween-20 per mL of culture medium. Following incubation on a rocking platform at 4°C for 20 h, the beads were collected by centrifugation, washed with 10 volumes of TST buffer (50 mM Tris•HCl pH 8.0, 150 mM NaCl, 0.05% Tween-20) and 2 volumes of 5 mM NH_4Ac pH 5.0. The recombinant ProtA-core 2 GlcNAc-T enzyme was then eluted with 1 volume 0.5 M acetic acid pH 3.4 and resuspended in 3 volumes of 0.5 M MES pH 7.5 (Calbiochem). One µU of enzyme activity is defined as the amount of protein forming 1 pmol/min of reaction product.

Solid phase Core 2 GlcNAc-T assay: A stock solution of the core 2 acceptor glycopolymer **3** was prepared by re-suspending the acceptor in water to a concentration of 1.25 mg/mL and then incubating the solution at 60°C for 1 h. The solution was gently mixed at 15 min intervals during this time to allow the polymer to unwind and become fully dissolved. The glycopolymer solution was not vortexed since strong agitation may cause shearing of the polymer backbone. Sodium azide (0.05%) was added as a preservative and the stock solution of glycopolymer was stored at room temperature. Wallac 96-well Printed Rigid Sample Plates (1450-511; Wallac, FI) were used in all cases for the solid phase assay. To prepare the plates for coating with acceptor, the wells were washed twice with 100 µl of methanol and then rinsed 3 times with 200 µl of water. After allowing the plates to dry at room temperature, the wells were coated with acceptor by adding 60 µl of a 33.3 µg/mL of glycopolymer solution and incubated overnight at room temperature. Following the incubation, unbound glycopolymer was removed by washing 3 times with 200 µl water and the remaining liquid in wells was allowed to evaporate by incubating the plates at 37°C (or room temperature) for approximately 1 h. Dried-coated plates could be used immediately or sealed and stored for use at a later date.

The HTS core 2 GlcNAc-T assay consisted of 20 µl of test compound, 20 µl of 3x assay buffer consisting of 90 mM MES pH 6.7, 10 mM EDTA (Sigma), 0.0075 mM UDP-GlcNAc (Sigma) and 0.1 µCi of UDP-[^{3}H]GlcNAc (16 Ci/mmol; Toronto Research Chemicals) and 20 µl of recombinant core 2 GlcNAc-T (containing 8-10 µU/µl) per reaction in 96 well plates. To minimize pipetting, the enzyme and the 3x buffer were routinely combined and 40 µl of the enzyme-buffer mix was added to the wells following the addition of the test compounds. After incubating the plates at 25°C for 60 min, the reactions were stopped by adding 175 µl of water to each well, aspirating the contents and washing 4 times with 190 µl water. The radioactive signal was measured using a MicroBeta plate counter (Wallac, FI) after adding 100 µl of OptiPhase Supermix scintillation cocktail (Wallac) to each well and incubating for >2 h, to allow for mixing. Each plate in HTS had 4 controls with vehicle added rather than test extracts, and background was determined with the omission of enzyme, also 4 wells per plate. Background was subtracted for each plate, and HTS results were expressed as a percentage of control reactions on the plate. The HTS assays were run on a Beckman integrated robotic platform using a Biomek 200 pipetting station and Zymark rotating robotic arm. PanLabs (Seattle, WA) supplied a collection of 30,000 bacterial and fungal extracts in 96 well plates.

The dried extracts were resuspended in DMSO, and diluted into water at 0.15% DMSO for the core 2 GlcNAc-T HTS.

Solution Phase core 2 GlcNAc-T assays. The core 2 GlcNAc-T solution phase assay mixture was similar to that used in earlier studies (Yousefi et al., J.Biol.Chem. 266:1772-1783, 1991; Williams et al., J.Biol.Chem. 255:11253-11261, 1980) but was adapted for automation on the Beckman robotic platform. For HTS assays, 10 µl of test extract, 10 µl of 3x assay buffer (90 mM MES pH 6.7, 10 mM EDTA, 3 mM Galβ1-3GalNAcα-pNp as acceptor, 3 mM UDP-GlcNAc (Sigma) and 0.1 µCi of UDP-[^{3}H]GlcNAc (16 Ci/mmol; Toronto Research Chemicals), and 10 µl of recombinant core 2 GlcNAc-T enzyme (4-5 µU activity) was added to wells of the titre plate. Reactions in a total volume 30 µl were incubated for 1-2 h at 37°C and stopped by adding 200 µl cold water. Plates were processed immediately or stored at -20°C. To recover the product, the assay mix was aspirated through C18 packed pipette tips (BGBS96C18 Biotips™, National Scientific) and the packing was then washed 3 times with 200 µl of H_2O. Bound product was eluted into ß-scintillation counting plates (Wallac 96-well Printed Rigid Sample Plate; 1450-511) by washing the C18 packing 3 times with 100 µl of 100% ethanol. The eluates were then dried overnight at room temperature to remove the ethanol and the radioactive signal was counted in a β-scintillation counter after the addition of counting fluid. Reaction products were found to accumulate in a linear manner for up to 2 hours of incubations. The C18 packed tips were cleaned and regenerated following the processing steps by washing once with 200 µl ethanol and then 3 times with 100 µl of H_20.

RESULTS

Glycopolymers for solid-phase glycosyltransferase assays:

The disaccharide acceptor Galß1-3GalNAcα-R where R is either octylmethyl or paranitophenyl has been used routinely in solution core 2 GlcNAc-T assays where UDP-[^{3}H]GlcNAc is the sugar-nucleotide donor. The product, Galß1-3([^{3}H]GlcNAcß1-6)GalNAcα-R is captured on C_{18} solid support, eluted with ethanol, and measured in a ß-counter (Yousefi et al, J.Biol.Chem. 266:1772-1783, 1991). This procedure has been miniaturized and automated, but remains relatively slow compared to high throughput (HTS) ELISA-style assays. The glycopolymers with Galß1-3GalNAcα-groups were prepared by chemical synthesis and reacted with recombinant ProtA-core 2 GlcNAc-T to establish the condition for the solid-phase glycosyltransferase assays. The water-soluble glycopolymer acceptors (**3-8**, **10**, and **11-14** Figure 9) used in the solid-phase glycosyltransferase assay are polyvalent substrates composed of N-substituted polyacrylamide backbones containing one disaccharide Galβ1-3GalNAcα-O$(CH_2)_3S(CH_2)_2$ (**2**) or trisaccharide [GlcNAc(β1-2)]Man(β1-6)Glc(β-O$(CH_2)_3S(CH_2)$) residues for every ten acrylamide backbone monomers. The viscosity-average molecular weight M_v of the core polymer was determined to be 42.1 kDa based on polyacrylamide derived from **1** by treatment with aqueous ammonium alone. The ratio of sugar to acrylamide of one to ten was determined using high field ^{1}H-NMR spectroscopy and was based on previous optimization experiments using analogous glycopolymers in enzyme-linked lectin assays (ELLA) (Roy, Trends in Glycoscience and Glycotech. 8:79-99 1996). The copolymer backbones were modified with various alkylamines to enhance their

lipophilicity and thus, increase their adsorption behaviors to the surface of the polystyrene microtiter plates.

Copolymers **6** and **14**, having the most lipophilic N-propylacrylamide backbones, were about eight times more sensitive than either N-ethyl (**5, 13**) or acrylamide (**3, 11**) copolymers while copolymers **4** and **12**, having an N-methyl substituent, were the least effective coating acceptor. Copolymer **10**, containing a shorter allyl spacer was also found unsuitable for the enzymatic glycosylation, presumably because of the inaccessibility of the GalNAc residues in the enzyme's active site. Similarly, copolymers 7 (co-biotin) or **8** (co-methacrylamide) provided either poor coating or poor enzymatic glycosylation. Biotin-containing copolymer **7** was initially designed to serve as coating substrate after capture by streptavidin/avidin pre-coating. Glycopolymer **6**, having the most lipophilic N-propylacrylamide backbone, was about eight times more effective than either N-ethyl (**5**) or acrylamide (**3**) glycopolymers while glycopolymer **4**, having an N-methyl substituent, was the least effective coating acceptor.

Coating plastic wells with glycopolymer. Early in the development of the solid-phase assay, variable results were observed with different batches of plastic plates. A number of pre-wash solutions were tested for their ability to improve the consistency of the core 2 GlcNAc-T reaction. Pre-washing the plastic with organic solvents improved the signal by 2-4 fold and eliminated variability between different lots of plates. Washing with non-ionic detergent reduced reaction efficiency. Based on these results, the 96 well plastic plates were routinely washed twice with methanol, then twice with water and stored dry prior to coating with the glycopolymer **3**. Washing of plastic plates was performed on the Beckman 2000 workstation with robotic arm. The time and temperature dependency for coating the wells with glycopolymer was determined, and overnight coating with 2 μg/mL at 20°C was determined to be optimal. Glycopolymer **3** was determined to be in excess, as the recovered solution of polymer was used to coat wells subsequently, and produce 80-90% of the reaction product realized with the first coating. However, glycopolymer solutions were routinely used only once.

Characterization of the Core 2 GlcNAc-T solid-phase assay In the initial experiments, 96 well plates were coated with a solution of 2 μg/well of glycopolymer acceptor **3**, and the reaction products were observed to be proportional to added enzyme over 60 minutes. However, the K_m for UDP-GlcNAc, and for acceptor using ProtA-core 2 GlcNAc-T in the solution assay was determined to be 1.75 mM and 146 μM respectively. In contrast, the solid-phase glycosyltransferase reaction conditions employ substrates well below K_m concentrations, as the amount of glycopolymer **3** bound to the plastic is limiting. Therefore, the sugar-nucleotide concentration is adjusted to optimize the detection of radioactive product and is also below K_m concentrations. To establish that the substrates are not exhausted during the 60 minute reaction, a time course and titration of UDP-GlcNAc was performed at 37°C. At higher concentrations of UDP-GlcNAc, the reaction went to completion in less than 5 minutes. However, with UDP-GlcNAc at 2.5 μM, and 200 μU of enzyme activity, the core 2 GlcNAc-T reaction product accumulated in a time-dependent manner for 30-60 minutes. The maximal product formed was 6-10 pmoles per well, and when 2.5 μM UDP-GlcNAc was used in the reaction, this represented approximately 4% utilization of the sugar-nucleotide donor. To simplify the HTS

protocol, these conditions (2.5 μM UDP-GlcNAc, 200 μU enzyme) were then further tested at room temperature (approximately 20°C). Under these conditions, product accumulation was found to be linear with time for approximately 60 min, and thus, the HTS assay was routinely performed at room temperature.

Glycopolymer acceptors for GlcNAc-TV were also made and tested using recombinant enzyme (Figure 10). Similar to that observed for core 2 GlcNAc-T, glycopolymer **14**, with the most lipophilic N-propylacrylamide backbone more effective than either N-ethyl (**13**) or acrylamide (**11**) glycopolymers. Glycopolymer **12**, an N-methyl substituent linker was the least effective coating acceptor. The GlcNAc-TV reactions product using glycopolymer **14** accumulated in a time and enzyme dependent manner.

Core 2 GlcNAc-T high-throughput screen (HTS) of microbial extracts. A microbial library of 30,000 extracts was subjected to HTS using the core 2 GlcNAc-T solid-phase assay as the primary screen (ie. glycopolymer **3**). Normalized results from a typical run of 1,600 assays are shown in Figure 11. The signal to noise was 20-fold and the CV of the positive controls was ±9.4 % assay. A series of 48 hit extracts, chosen from the total primary screen data (ie. >50% inhibition) were placed on plates with 88 other inactive extracts, and re-tested in a 5 point dilution series using both the solid-phase and solution core 2 GlcNAc-T assays. 94.4 % (17/18) of the hits identified in the solution assay were also hits in the solid phase assay. Additional hits, 3 with good and 8 with inconclusive titration curves were observed in the solid-phase assay. Active extracts identified in the core 2 GlcNAc-T HTS can be fractionated to identify active molecules for further testing in cell culture and animal models of disease.

DISCUSSION

A solid phase glycosyltransferase assay was optimized for use with recombinant core 2 GlcNAc-T. The assay was also tested with glycopolymer acceptors for GlcNAc-TV and GlcNAc-TI and shown to be acceptable for other glycosyltransferase enzymes. The solid-phase core 2 GlcNAc-T assay was used in a HTS of a library of microbial extracts and active extracts were confirmed with a high degree of concordance in the solid-phase and a conventional solution assay. The solid-phase assay format allowed 5-6 fold increase in throughput compared to a solution phase assay, for a rate of 7,500 per day.

The core 2 GlcNAc-T HTS of 30,000 microbial extracts yielded hits at a frequency of 1.5% in the solution assay. One third of the library was also screened using the solid phase assay and yielded a high degree of concordance with the results previously found with the solution assay.

EXAMPLE 3

High Throughput L-PHA Assay

Materials and Methods

Chemicals. L-PHA, Triton X-100 and *para*-nitrophenylphosphate were obtained from Sigma; diethanolamine was purchased from Fisher.

Cells. The origin and properties of the DBA-2 strain lymphoreticular tumor MDAY-D2 have been previously described (Kerbel, RS, Florian, M, Man, MS, Dennis, J and McKenzie IF (1980) *J.Natl.Cancer Inst.*, 64, 1221-1230). Cells were cultured in α-modified Eagle's medium containing 2%

heat-inactivated fetal calf serum (Gibco BRL) at 37°C in a 95%O_2/5%CO_2 humidified atmosphere.

Alkaline phosphatase assay. Determinations were carried out using 96-well plates. Each well contained a variable number of MDAY-D2 cells maintained in 125 µl of culture medium supplemented with 2% fetal calf serum. The alkaline phosphatase reaction was initiated by adding 75 µl of assay mixture (1 M diethanolamine buffer, pH 9.8, 2 mM $MgCl_2$, 1% Triton X-100 and 2.5 mM *para*-nitrophenylphosphate) and incubated at 37°C for up to 90 min. The reaction was stopped with 80 µl of 3.5 M NaOH. After 15-30 min of colour development, absorbance of the chromogenic product *para*-nitrophenol was measured at 405 nM using a multiwell scanning photometer (Thermomax Multiplate Reader, Molecular Devices). Background values were determined through assays performed on culture medium alone in the absence of cells and routinely subtracted. Linearity between the absorbance at 405 nM and concentration of *para*-nitrophenol was in the range 0-2.5 (ε = 17.23 $mM^{-1}cm^{-1}$).

Screening via L-PHA assays. The procedure was completely automated by using a robotic workstation (Biomek 2000, Beckman) capable of processing nine 96-well plates simultaneously. Determinations were performed in flat bottom 96-well plates (88 samples + 8 controls per plate). Each well (columns 1-11) received 10 µl of compound (in 2.5% DMSO), while 10 µl of 2.5% DMSO in water was added to column 12. All 96 wells received $5x10^3$ MDAY-D2 cells in 90 µl culture medium supplemented with 2% FCS. After 16-20 h incubation at 37°C, 25 µl of L-PHA (100 µg/mL in culture medium) was added to the first 11 columns and to 4 wells of the 12th (positive control). The other 4 wells received 25 µl of medium supplemented with 2% FCS (negative control). Assay plates were maintained for 30-36 h at 37°C, and alkaline phosphatase activity was measured according to the protocol described above using an incubation time of 1 h. Cell density was subconfluent throughout the course of the assay. Proliferation indices were expressed as percentage values, calculated with the formula:

Normalized Signal = (A_{405} of sample - mean A_{405} positive control)/(mean A_{405} negative control - mean A_{405} positive control)

RESULTS

In homogeneous screening assays the results are determined without washing or transferring target proteins or cells, reactants, and test compounds from the assay plates. Homogeneous assay formats save time in performing the assay, and with less manipulation, the low errors are observed. This translates into fewer follow-up assays on putative hits in a large screen. A homogeneous cell-based assay has been developed that measures cell growth and variability using endogenous alkaline phosphatase activity.

MDAY-D2 tumor cells maintained in tissue culture in log phase of growth exhibit alkaline phosphatase activity in the range 40-80 nmol/h/10^4 cells. Alkaline phosphatase measurements were linear over time for at least 90 min, and directly proportional to cell number, allowing detection of 1500 cells. MDAY-D2 doubling time calculated through accumulation of alkaline phosphatase activity

was ~14h, similar to that measured by counting cells. The alkaline phosphatase assay is comparable in reproducibility and sensitivity, with a commercially available, chemiluminometric method.

The apparent Km of alkaline phosphatase measured in the MDAY-D2 whole cell assay was 0.86 mM, whereas the value exhibited by the soluble enzyme present in fetal calf serum was 0.21 mM. Background activity present in culture medium containing 2% FCS produced an A_{405} of 0.2 after 1h of incubation and represented approximately 10% of the signal with the standard assay conditions.

Swainsonine blocks α-mannosidase II, acting as an inhibitor of complex-type N-oligosaccharide biosynthesis resulting in resistance to the toxicity of L-PHA lectin. Swainsonine protected MDAY-D2 cells from L-PHA toxicity with an IC_{50} value of 0.0528 +/- 0.0087 μM (n =8). An IC_{50} value of 0.2 μM was previously reported using thymidine incorporation as a measure of cell growth (Dennis et al, 1993, Biochem. Pharmacol., 46, 1459-1466).

The alkaline phosphatase cell assay was applied to high-throughput screening of a microbial extract library. The signal to noise ratio (i.e. growth of L-PHA-treated/control MDAY-D2 cells) was 5 and the coefficient of variation of both negative and positive control samples was 4.2% and 2.4%, respectively. Twenty microbial extracts of the 30,000 tested increased cell viability in the presence of L-PHA to a degree greater than 3xSD of the mean. These fell on the right-hand side of normal distribution Figure 12). On re-testing, 4 of the 20 extracts were confirmed as hits for further fractionation. A number of extracts suppressed growth below that observed in the presence of L-PHA (i.e. left of the normal distribution). These likely contain compounds that are generally toxic, and not of interest.

DISCUSSION

The proliferation rate of MDAY-D2 cells was monitored by employing an assay of alkaline phosphatase activity. The motivation for this work was to establish a simple, reproducible and cost-effective procedure to be applied for high throughput screenings via L-PHA assay. A colorimetric determination of alkaline phosphatase activity has been found suitable to measure lymphokine-dependent B cell proliferation (Hashimoto N and Zubler RH (1986) *J.Immunol.Methods* 90, 97-103.); the advantage of the protocol described herein is that the method can be carried out in a single step, without removal of the culture medium or cell pelletting and washing, thereby permitting fully automated procedures. Furthermore, use of a robotic platform allowed simultaneous processing of thirty-six 96-well plates. The method is very cost-effective, especially when compared to other commercially available assay kits.

Sensitivity and accuracy of the alkaline phosphatase method are based upon several observations: i) MDAY-D2 cells express relatively high levels of enzyme, whereas background activity present in fetal calf serum (2%) is low; ii) readings of A_{405} were found to be proportional to the concentration of reaction product; iii) the reaction is linear with time within a relatively wide interval of up to 1.5h; and, iv) the numbers of MDAY-D2 cells (both untreated and L-PHA-treated) correlated with enzyme activity within a relatively wide range (i.e. $1x10^3$ up to $2.5x10^5$ cells). The assay was performed using 1mM substrate (final concentration), 4-fold above the Km of the serum enzyme (i.e. 0.21 mM) and similar to that of the cellular enzyme (i.e. 0.86 mM). With this 4-fold difference in Km

values, the signal to background ratio may be amplified by increasing the substrate concentration above 1mM.

The assay was proven reliable by several indicia. For example, when swainsonine, a known inhibitor of N-linked oligosaccharide processing, was employed in conjunction with the L-PHA/alkaline phosphatase assay, the IC_{50} of the drug compared well with that previously reported using thymidine incorporation to measure cell growth. Additionally, the results of the cell proliferation measurements corresponded to those obtained using another single-step, commercially available chemiluminometric kit. Finally, during the screening of extract libraries (30,000 samples), control measurements of alkaline phosphatase activity (n=3200) showed coefficient of variations which were markedly low.

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

Table 1

Enzyme	Donor	Acceptor
Glycoprotein-fucosylgalactoside alpha-N-acetylgalactosaminyltransferase (EC 2.4.1.40)	UDP D-GalNAc	Glycoprotein α-L fucosyl-(1,2)-D-galactose
(N-acetylneuraminyl)-galactosylglucosylceramide N-acetyl galactosaminyltransferase (EC 2.4.1.92)	UDP D-GalNAc	(N-acetylneuraminyl)D-galactosyl - D-glucosylceramide
β-1,4-N-acetyl galactosaminyl transferase (murine T-lymphocyte CT antigen synthesis) (Swiss Prot Q09199)	UDP D-GalNAc	(N-acetylneuraminyl)D-galactosyl-D-R
N-acetyllactosaminide alpha-1,3 galactosyltransferase (galactosyl transferase) (EC 2.4.1.151)	UDP-D-Gal	β-D-galactosyl-(1,4) N-acetyl-D-glucosaminyl-R
Glycoprotein-fucosylgalactoside –α –galactosyltransferase (EC 2.4.1.37)	UDP-D-Gal	Glycoprotein α-L fucosyl (1,2)-D-galactose
N-acetyllactosamine synthase (EC 2.4.1.90)	UDP-Gal	N-acetyl-D-glucosamine
2-hydroxyacyl sphingosine 1- -galactosyltransferase (EC 2.4.1.45)	UDP-Gal	2-(2-hydroxyacyl) sphingosine
UDP-galactose-glucose galactosyltransferase N Acetyllactosamine synthase	UDP-D-Gal	D-Glucose
Fucosylglycoprotein α-N-acetylgalactosaminyl transferase	UDP-D-GalNAc	Glycoprotein α-L-fucosyl-(1,2)-D-galactose
Galactoside-2-L-fucosyl transferase (EC 2.4.1.69)	GDP-L-fucose	β-D-galactosyl-R
galactoside 3(4)-L fucosyl - transferase (EC 2.4.1.65); fucosyltransferase 6 (SWISS PROTP51993); fucosyltransferase 5 (SWISS PROT Q11128)	GDP-L-fucose	1,3-β-D-galactosyl N- acetyl-D-glucosaminyl-R
α-1,3-mannosyl-glycoprotein β-1,2 N-acetylglucosaminyl transferase (GnT I) (EC 2.4.1.101)	UDP-D-GlcNAc	α-D-mannosyl-1,3 (R1)- β-D-mannosyl-R2

Enzyme	Donor	Acceptor
α-1,6-mannosyl-glycoprotein-β 1,2 N-acetylglucosaminyl transferase (GnT II) (EC 2.4.1.143)	UDP-D-GlcNAc	α-D-mannosyl-1,6 (N-acetyl— β-D glucosaminyl-1,2-α- D-(mannosyl-1,3-)-β–D- mannosyl-R
α-1,3(6)-mannosylglycoprotein β 1,6-N-acetyl-glucosaminyl transferase (EC 2.4.1.155) (GnT V)	UDP-D-GlcNAc	N-acetyl-β-glucosaminyl-1,2 alpha-D-mannosyl-1,3(6)-(N-acetyl-β-D-glucosaminyl-1,2-α-D-mannosyl-1,6(3)-βD-mannosyl-1,4-N-acetyl-β-D-glucosaminyl-R
Polypeptide-N-acetylgalactosaminyl transferase (EC 2.4.1.41)	UDP-D-GalNAc	Polypeptide
β-1,4-mannosyl-glycoprotein β-1,4-N-acetylglucosaminyl transferase (GnT III) (EC 2.4.1.144)	UDP-D-GlcNAc	N-acetyl-β-D-glucosaminyl-1,2-alpha-D-mannosyl-1,3-(N-acetyl-β-D-glucosaminyl-1,2-α-D-mannosyl-1,6)-β-D-mannosyl-1,4-N-acetyl-β-D-glucosaminyl-R
Chitin synthase (EC 2.4.1.16)	UDP-D-GlcNAc	{(1,4)-(N-acetyl-β-D-glucosaminyl)} (N)
β-1,3-galactosyl-O-glycosyl glycoprotein-β1,6-N acetylglucosaminyltransferase (EC 2.4.1.102) (β1,6 (O-linked, core 2))	UDP-D-GlcNAc	β-D-galactosyl-1,3 N-acetyl-D-galactosaminyl-R
UDP-N-acetylglucosamine-dolichyl-phosphate N-acetyl-glucosaminephosphotransferase (EC 2.7.8.15)	UDP-D-GlcNAc	Dolichyl phosphate
Galactoside 3-fucosyltransferase (EC 2.4.1.152)	GDP-L-fucose	1,4-β-D-galactosyl-N-acetyl-D-glucosaminyl-R
Fucosyltransferase 7 (SWISS PROT Q11130)	GDP-L-fucose	α-2,3-Neu-N-acetyl-1,4-β-D-galactosyl-N-acetyl-D-glucosaminyl-R

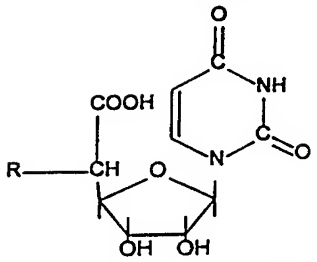
Table 2

3-deoxy-D-manno-octulosonic acid (KDO) transferase
Chlamydia pneumoniae KDO transferase gb:z31593
Chlamydia psittaci KDO transferase gb:x80061
Chlamydia psittaci gseA transferase gb:x69476
Chlamydia trichomatis KDO transferase gb:m64618
Chlamydia trachomatis gseA transferase gb:z22653 gb:z22654 gb:z22655 gb:z22656 gb:z22659
Escherichia coli kdtA gb:m60670 gb:m86305 gb:u00039 sw:p23282 (kdta_ecoli)
Haemophilus influenzae kdtA gb:l45293 gb:u32748 sw:p44806 (kdta_haein)
O-antigen Gal-2--fucosyltransferase
Yersinia enterocolitica fucosyltransferase gene gb:u18674 gb:u25113 gb:u46859
cld (chain length determining) (similar to putative undecaprenyl-P--GlcNAc transferase)
Escherichia coli cld1 gb:z17241 sw:q05032 (cld1_ecoli)
Escherichia coli cld2 gb:m89934 sw:p35272 (cld2_ecoli)
Salmonella typhimurium cld gb:z17278 sw:q04866 (cld_salty)
Shigella flexneri cld gb:x71970 sw:p37792 (cld_shifl)
cpsD galactosyltransferase
Streptococcus agalactiae cpsD gene gb:l09116
lgtA galactosyltransferase
Rhizobium leguminosarum Allaway et al (1996) unpublished gb:x94963
murG N-acetylglucosaminyltransferase
Bacillus subtilis murG gene gb:s56399 gb:x64259
mraY phospho-N-acetylmuramoylpentapeptide synthase EC 2.7.8.13
Bacillus subtilis mraY gene gb:z15056 sw:q03521 (mray_bacsu)
Escherichia coli mraY gene gb:x51584 gb:x55034 gb:d10483 sw:p15876 (mray_ecoli)
mtfA, mtfB, mtfC mannosyltransferases
Escherichia coli mtfA, mtfB and mtfC genes gb:d13231 gb:d43637
neuS 2,8-sialyltransferase
Escherichia coli neuS gene gb:x60598
nodC N-acetylglucosaminyltransferase
Azotorhizobium caulinodans gb:l18897 sw:q07740 (nodc_azoca)
Bradyrhizobium elkanii gb:u04609
rfaB 1,6-galactosyltransferase
Escherichia coli rfaB gene gb:m80599 gb:u00039 sw:p27127 (rfab_ecoli)
Salmonella typhimurium rfaB gene gb:s56361 sw:q06994 (rfab_salty)
rfaC heptulosyltransferase 1
Escherichia coli rfaC gene gb:u00039 sw:p24173 (rfac_ecoli)
rfaG glucosyltransferase
Escherichia coli rfaG gene sw:p25740 (rfag_ecoli)
rfaI 1,3-galactosyltransferase EC 2.4.1.44
Escherichia coli rfaI gene gb:m80599 gb:u00039 sw:p27128 (rfai_ecoli)
Salmonella typhimurium rfaI gene gb:x53847 sw:p19816 (rfai_salty)

Table 2 Cont'd

rfaJ 1,2-glucosyltransferase EC 2.4.1.58
Escherichia coli rfaJ gene sw:p27129 (rfaj_ecoli)
Salmonella typhimurium rfaJ gene sw:p19817 (rfaj_salty)
rfaK 1,2-N-acetylglucosaminyltransferase EC 2.4.1.56
Escherichia coli rfaI gene gb:u00039 sw:p27242 (rfai_ecoli)
rfbF galactosyltransferase
Campylobacter hyoilei rfbF gene gb:x91081
Klebsiella pneumoniae rfbF gene gb:l31762 gb:l41518
rfbN rhamnosyltransferase
Salmonella typhimurium rfbN gene gb:x56793
rfbP galactosyltransferase
Yersinia enterocolitica rfbP gene gb:u18674 gb:u25113 gb:u46859
Salmonella enterica rfbP gene gb:x61917
rfbQ rhamnosyltransferase
Salmonella enterica rfbQ gene gb:x61917
rfbU mannosyltransferase
Salmonella typhimurium rfbU gene gb:x56793 sw:p26402 (rfbu_salty)
rfbW second mannosyltransferase
Salmonella enterica rfbW gene gb:x61917
rfbZ first mannosyltransferase
Salmonella enterica rfbZ gene gb:x61917
rfe undecaprenyl-P--GlcNAc transferase
Escherichia coli rfe gene gb:s75640 gb:m87049 gb:m76129 sw:p24235 (rfe_ecoli)
Mycobacterium leprosum rfe gene gb:u15186 sw:p45830 (rfe_mycle)
rffM probable N-acetyl-D-mannosaminuronic acid transferase
Escherichia coli rffM gene gb:m87049 sw:p27836 (rffm_ecoli)
Salmonella typhimurium rffM gene gb:m95047 sw:p37457 (rffm_salty)
rffT probable 4--fucosyltransferase
Escherichia coli rffT gene gb:m87049 sw:p27835 (rfft_ecoli)
Salmonella typhimurium rffT gene gb:m95047 sw:p37458 (rfft_salty)
RhlAB rhamnosyltransferase
Pseudomonasaeruginosa rhlAB gene gb:l28170
Glycosyltransferase locus of *N. gonorrhoeae*
U.S. Patent No. 5,703,367 to Gotschlich

Table 3



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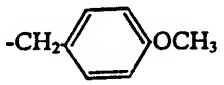
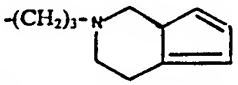
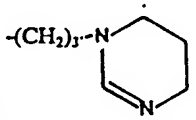
B	-NHCOR[1]	-CHR[3]R[4]	H	
C	-NHCOR[1]	-CHR[3]R[4]	H	
D	-NHCOR[1]	-CHR[3]R[4]	H	
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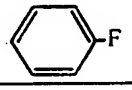
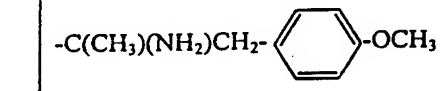
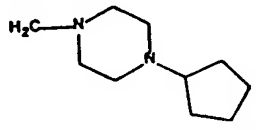
F	-NHCOR[1]	-CHR[3]R[4]	-NH_2	
G	-NHCOR[1]		-	-
H	-NHCOR[1]	-CHR[3]R[4]	-NH_2	
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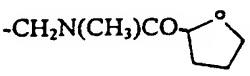
J	-NHCOR[1]	-CHR[3]R[4]	-NH_2	
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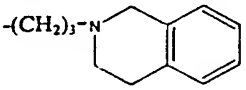
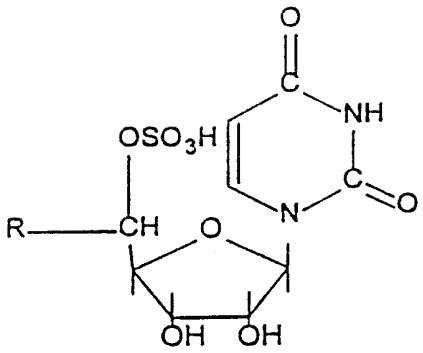
V	-NHCOR[1]	-CHR[3]R[4]	H	

Table 4



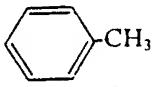
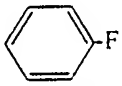
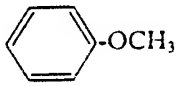
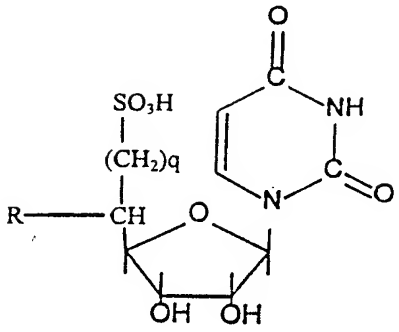
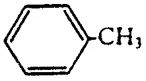
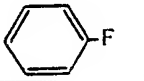
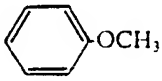
No.	R	R^1	R^2	R^4
K	-NHCOR^1	-CHR^{3}R^4	-NH_2	
L	-NHCOR^1	-CHR^{3}R^4	-NH_2	
M	-NHCOR^1	-CHR^{3}R^4	-NH_2	
N	-NHCOR^1	-CHR^{3}R^4	-NH_2	-$CH_2N(C_2H_5)CH_2CH(CH_3)OH$
O	-NHCOR^1	-CHR^{3}R^4	-NH_2	–$CH_2NHCOCH(CH_3)_2$

Table 5



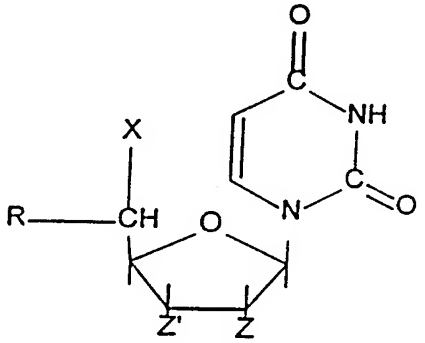
q = 0 or 1

No.	R	R^1	R^3	R^4
P	-NHCOR[1]	-CHR[3]R[4]	$-NH_2$	
Q	-NHCOR[1]	-CHR[3]R[4]	$-NH_2$	
R	-NHCOR[1]	-CHR[3]R[4]	$-NH_2$	
S	-NHCOR[1]	-CHR[3]R[4]	$-NH_2$	$-CH_2N(C_2H_5)CH_2CH(CH_3)OH$
T	-NHCOR[1]	-CHR[3]R[4]	$-NH_2$	$-CH_2NHCOCH(CH_3)_2$

WE CLAIM:

1. A combinatorial library comprising a predetermined collection of nucleoside peptide molecules for inhibiting the transfer of a sugar from a selected sugar nucleotide donor to a selected acceptor by a carbohydrate processing enzyme wherein a nucleoside peptide molecule comprises (a) a nucleoside monomer; (b) a spacer monomer coupled to the nucleoside monomer wherein the spacer monomer comprises one or more amide linked amino acid residues or mimetics thereof; and (c) cap monomers attached to the spacer monomer; wherein the nucleoside peptide molecules differ from each other as to the identity of at least one element of the nucleoside monomer, spacer monomer or cap monomers.
2. A combinatorial library as claimed in claim 1 wherein the carbohydrate processing enzyme is a glycosyltransferase involved in the biosynthesis of glycoproteins, glycolipids, or glycosyl phosphatidyl inositols.
3. A combinatorial library as claimed in claim 2 wherein the carbohydrate processing enzyme is an N-acetylglucosaminyltransferase I, II, II, IV, or V, or β-1,3-galactosyl-O-glycosyl-glycoprotein β 1,6-N-acetylgucosaminyl transferase (core 2 GlcNAc).
4. A combinatorial library as claimed in claim 1, 2, or 3 wherein the nucleoside monomer is uridyl, 2'-deoxyuridyl, or 5'-amino-5'deoxy-2',3'-O-isopropylidine uridyl.
5. A combinatorial library as claimed in any one of the preceding claims wherein the cap monomer is methyl (Me), formyl (CHO), ethyl (Et), acetyl (Ac), t-butyl (t-bu), anisyl, trifluoroacetyl (Tfa), benzoyl (Bz), 4-methylbenzyl (Meb), thioanizyl, thiocresyl, benzyloxymethyl, 4-nitrophenyl (Pnp), benzyloxycarbonyl (Z), 2-nitrobenzoyl (NBz), 2-nitrophenylsulphenyl (Nps), 4-toluenesulphonyl (Tosyl, Tos), pentafluorophenyl (Pfp), diphenylmethyl (Dpm), 2-chlorobenzyloxycarbonyl (Cl-Z), 2,4,5-trichlorophenyl, 2-bromobenzyloxycarbonyl (Br-Z), triphenylmethyl (Trityl, Trt), 2,2,5,7,8-pentamethyl-chroman-6-sulphonyl (Pmc), t-butyloxycarbonyl (Boc), benzyl (Bzl), benzyloxymethyl (Bom), and 9-fluorenylmethyloxycarbonyl (Fmoc).
6. A combinatorial library as claimed in any one of the preceding claims wherein the spacer monomer is a single amide linked amino acid, an amide linked dipeptide, or an amide linked tripeptide, or a mimetic thereof.
7. A nucleoside peptide molecule comprising a nucleoside monomer; a spacer monomer coupled to a nucleoside monomer, wherein the spacer monomer comprises one or more amide linked amino acid residues, or a mimetic thereof; and cap monomers attached to the spacer monomer.
8. A nucleoside peptide molecule of the formula I:

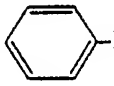
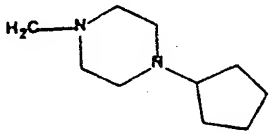
I

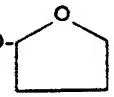
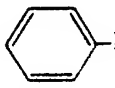
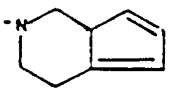
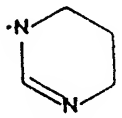
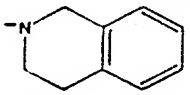
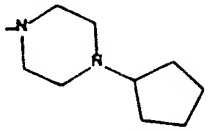


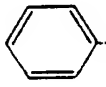
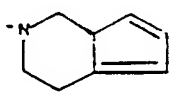
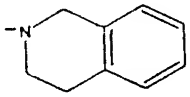
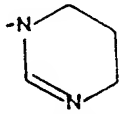
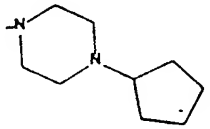
where X is H, –COOH, -OSO_3H, or $(CH_2)qSO_3H$ where q is 0 or 1, and R represents $(Y)_m$ where Y is an amide linked amino acid residue and m is 1-3, Z' and Z are the same or different and represent hydroxyl or alkoxy, or Z' and Z together form an acetonide group, and wherein free NH_2 groups in the compound of the formula I are capped with a cap monomer.

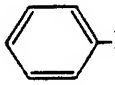
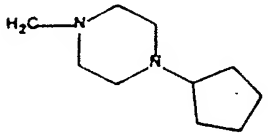
9. A nucleoside peptide molecule of the formula I as claimed in claim 8 wherein X is H, –COOH, -OSO_3H, or $(CH_2)qSO_3H$ where q is 0 or 1, Z and Z' are both hydroxyl or together form an acetonide group, R represents $-NHCOR^1$, wherein R^1 represents

(a) $-C(CH_3)(NH_2)CH_2-$  $-R^2$, wherein R^2 is alkoxy; or

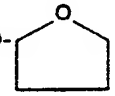
(b) $-CHR^3R^4$ wherein R^3 is hydrogen or $-NH_2$, and R^4 is  $-R^5$ wherein R^5 is halogen, alkyl, or alkoxy,  , $-CH_2N(CH_3)CH_2CH_2R^6$ or $-N(CH_3)CH_2CH_2R^6$ wherein R^6 is halogen,

$-CH_2N(CH_3)CO-$  , $-CH_2N(C_2H_5)CH_2CH(CH_3)OH$, or $-CH_2NHCOCH(CH_3)_2$, or R^4 represents $(CH_2)_nR^8$ wherein n = 0 to 5, R^8 is halogen,  $-R^9$ wherein R^9 is alkoxy,  ,  ,  ,  , $-N(CH_3)CH_2CH_2R^{10}$ wherein R^{10} is halogen, $-N(C_2H_5)CH_2CH(CH_3)OH$, or $-NHCOCH(CH_3)_2$ and wherein free amino groups are protected with a cap monomer.

10. A nucleoside peptide molecule of the formula I as claimed in claim 8 wherein X is –COOH, and R represents $-NHCOR^1$ wherein R^1 represents $-CHR^3R^4$ wherein R^3 is hydrogen, and R^4 is $(CH_2)_nR^8$ wherein n = 0 to 5, preferably 1 to 4, R^8 is halogen,  $-R^9$ wherein R^9 is alkoxy, halogen, or alkyl,  ,  ,  ,  , or $-N(CH_3)CH_2CH_2R^{10}$ wherein R^{10} is halogen, $-N(C_2H_5)CH_2CH(CH_3)OH$, or $-NHCOCH(CH_3)_2$.

11. A nucleoside peptide molecule of the formula I as claimed in claim 8 wherein . X is –COOH, and R represents –NHCOR^1 wherein R^1 represents –CHR^{3}R^4 wherein R^3 represents –NH$_2$, and R^4 is  –R^5 wherein R^5 is halogen, alkyl or alkoxy, 

-CH$_2$N(CH$_3$)CH$_2$CH$_2$R^6 wherein R^6 is halogen, -CH$_2$N(C$_2$H$_5$)CH$_2$CH(CH$_3$)OH, –CH$_2$NHCOCH(CH$_3$)$_2$

or -CH$_2$N(CH$_3$)CO- 

12. A nucleoside peptide molecule of the formula I as claimed in claim 8 wherein X is -OSO$_3$H, or (CH$_2$)qSO$_3$H where q is 0 or 1, R represents –NHCOR^1 wherein R^1 represents –CHR^{3}R^4 wherein R^3 represents –NH$_2$, and R^4 is

 -R^5 wherein R^5 is halogen, alkyl, or alkoxy, -CH$_2$N(C$_2$H$_5$)CH$_2$CH(CH$_3$)OH, or

–CH$_2$NHCOCH(CH$_3$)$_2$.

13. A process for preparing a combinatorial library containing a predetermined collection of nucleoside peptide molecules for inhibiting the transfer of a sugar from a selected sugar nucleotide donor having a heterocyclic amine base, to a selected acceptor by a carbohydrate processing enzyme comprising:

(a) coupling one or more amino acids, or mimetics thereof to a nucleoside monomer unit which comprises a heterocyclic amine base coupled to a sugar wherein the base corresponds to the heterocyclic amine base of the sugar nucleotide donor, or a modified form or analogue of the base; and

(b) capping any free functional groups or amine groups with a cap monomer unit.

14. A method of using a combinatorial library as claimed in claim 1 for screening for pharmacologically active molecules.

15. A solid-phase bioassay for identifying a compound having inhibitory activity against a carbohydrate processing enzyme which comprises (a) coupling an acceptor for the carbohydrate processing enzyme to a polymer and coating onto a carrier; (b) adding a carbohydrate processing enzyme, a sugar nucleotide donor labeled with a detectable substance, and a test compound; (c) measuring the detectable change produced by the detectable substance; and (d) comparing to a control in the absence of the test compound wherein a decrease in the amount of detectable substance with the test compound indicates that the test compound has inhibitory activity against the enzyme.

16. A method for identifying a compound that inhibits N-linked oligosaccharide processing comprising (a) reacting a test compound with cells expressing N-linked oligosaccharides in the presence of L-PHA and measuring alkaline phosphatase activity; and (b) comparing to a control in the absence of the compound wherein an increase in alkaline phosphatase activity indicates that the compound inhibits N-linked oligosaccharide processing.
17. A pharmaceutical composition containing a compound identified by a method as claimed in any one of claims 14, 15, or 16.

FIGURE 1


NH
O
O
N
O
O
O
CH3
H3C
HO
2,2-Dimethoxy propane
Acetone, CSA
NH
O
O
N
O
OH
HO
HO

FIGURE 2

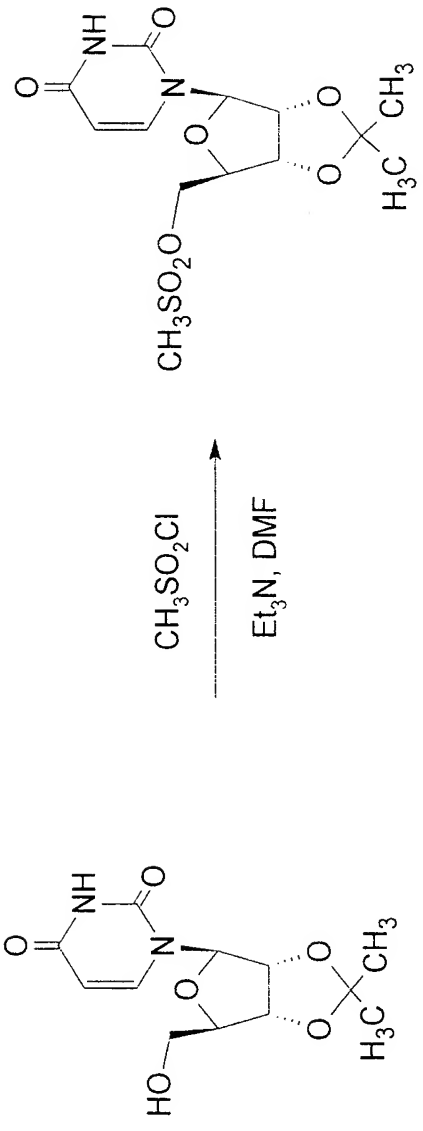


FIGURE 3

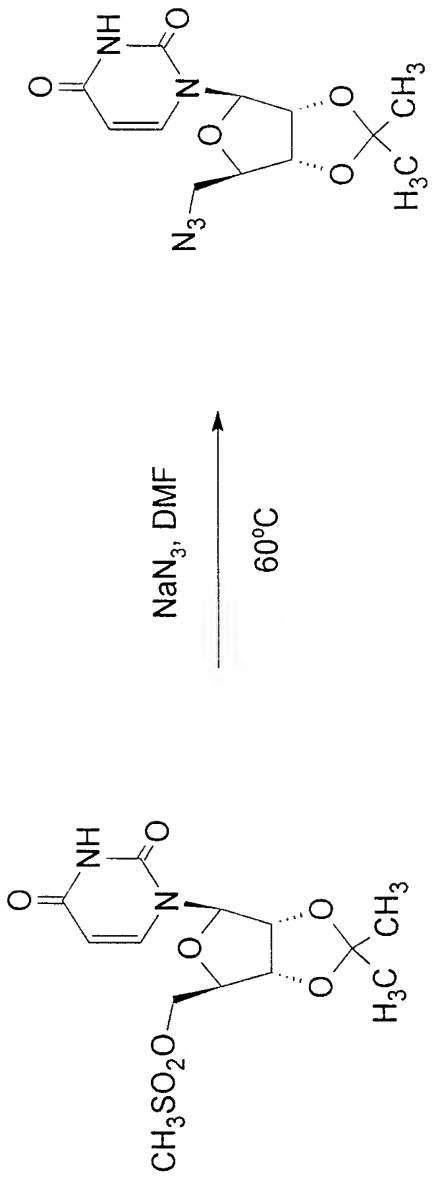


FIGURE 4

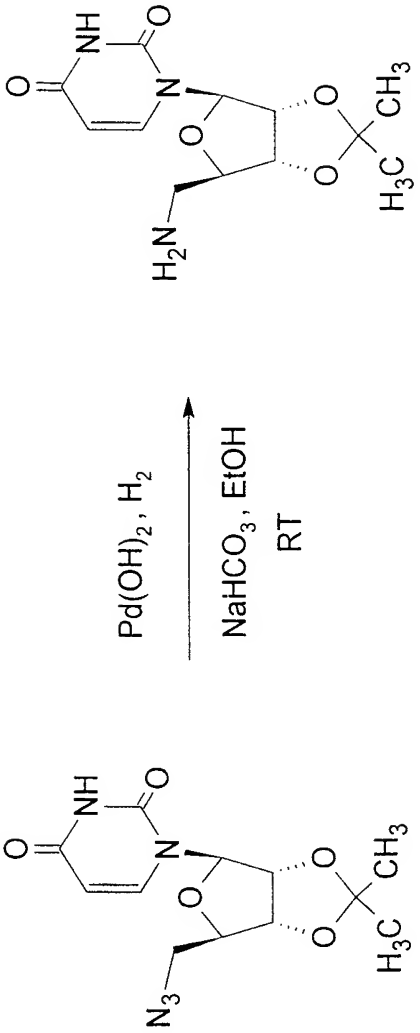

NH
O
O
N
O
O
O
CH3
H3C
H2N
Pd(OH)2, H2
NaHCO3, EtOH
RT
NH
O
O
N
O
O
O
CH3
H3C
N3

FIGURE 5

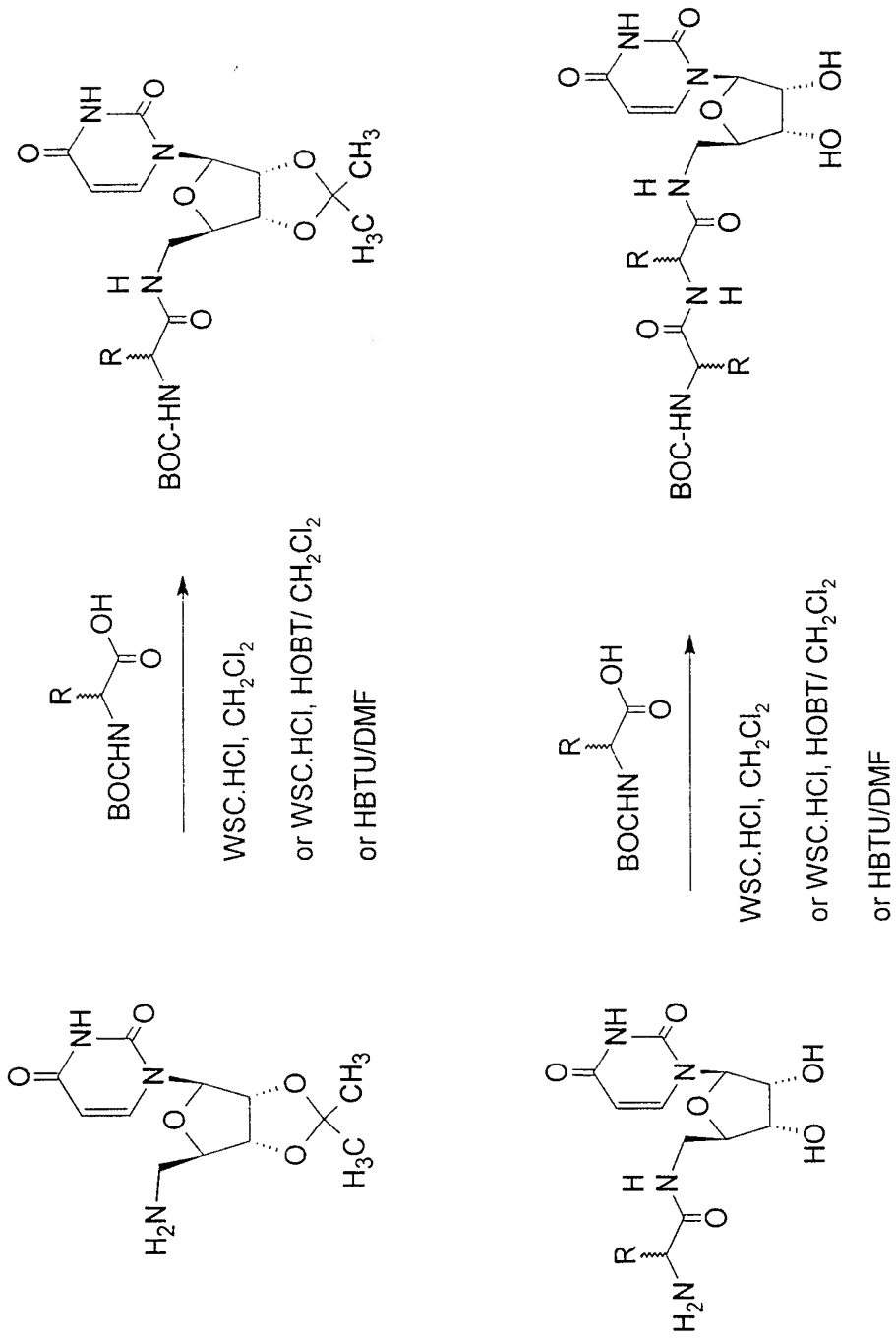

BOC-HN
BOCHN
WSC.HCl, CH_2Cl_2
or WSC.HCl, HOBT/ CH_2Cl_2
or HBTU/DMF
H_2N
H_3C
CH_3
HO
OH

FIGURE 6

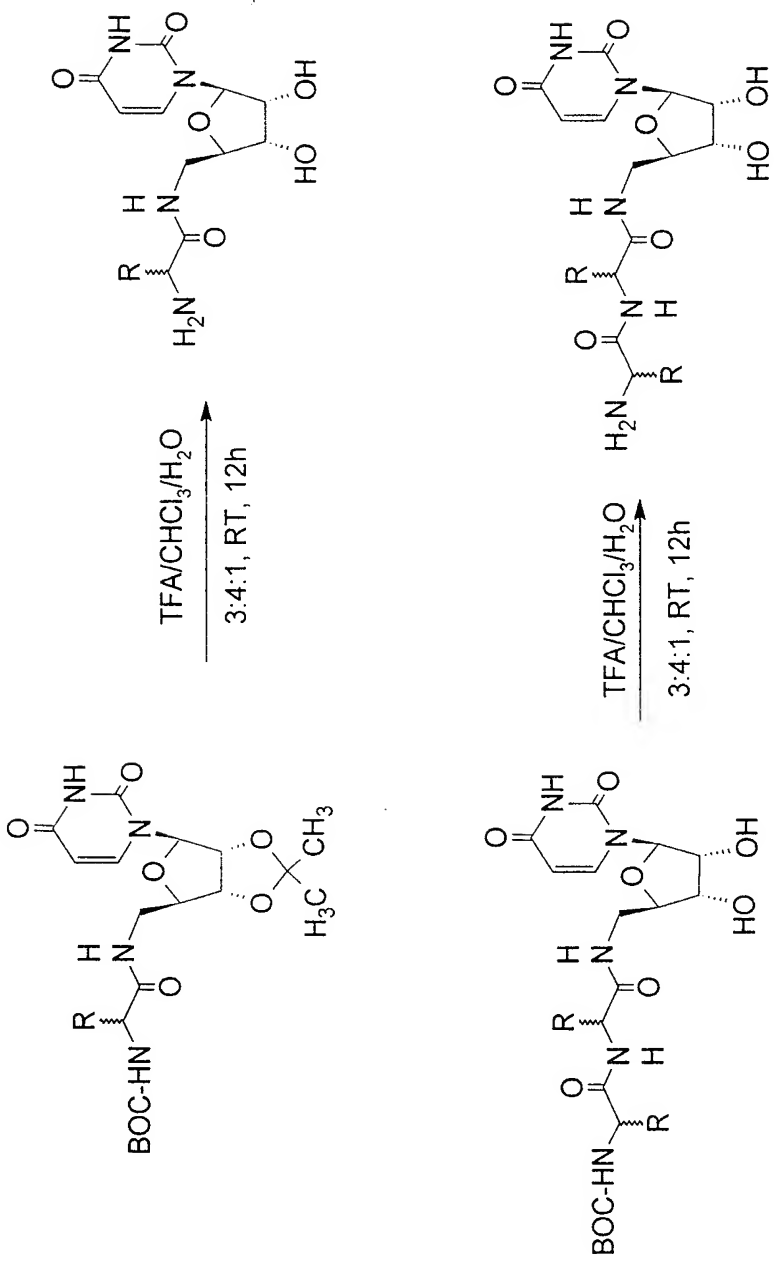


FIGURE 7

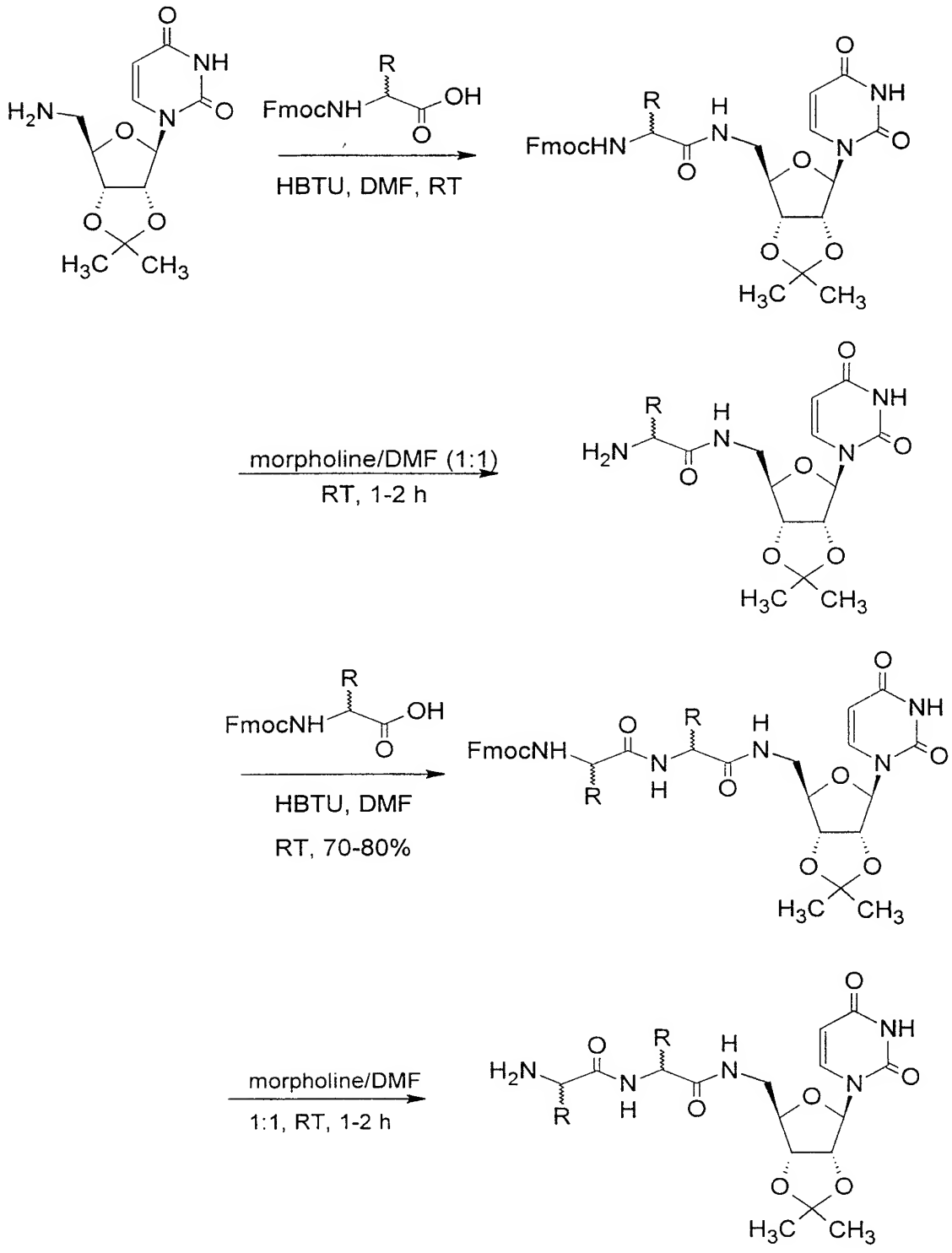

FmocNH
OH
HBTU, DMF, RT
morpholine/DMF (1:1)
RT, 1-2 h
HBTU, DMF
RT, 70-80%
morpholine/DMF
1:1, RT, 1-2 h

FIGURE 8

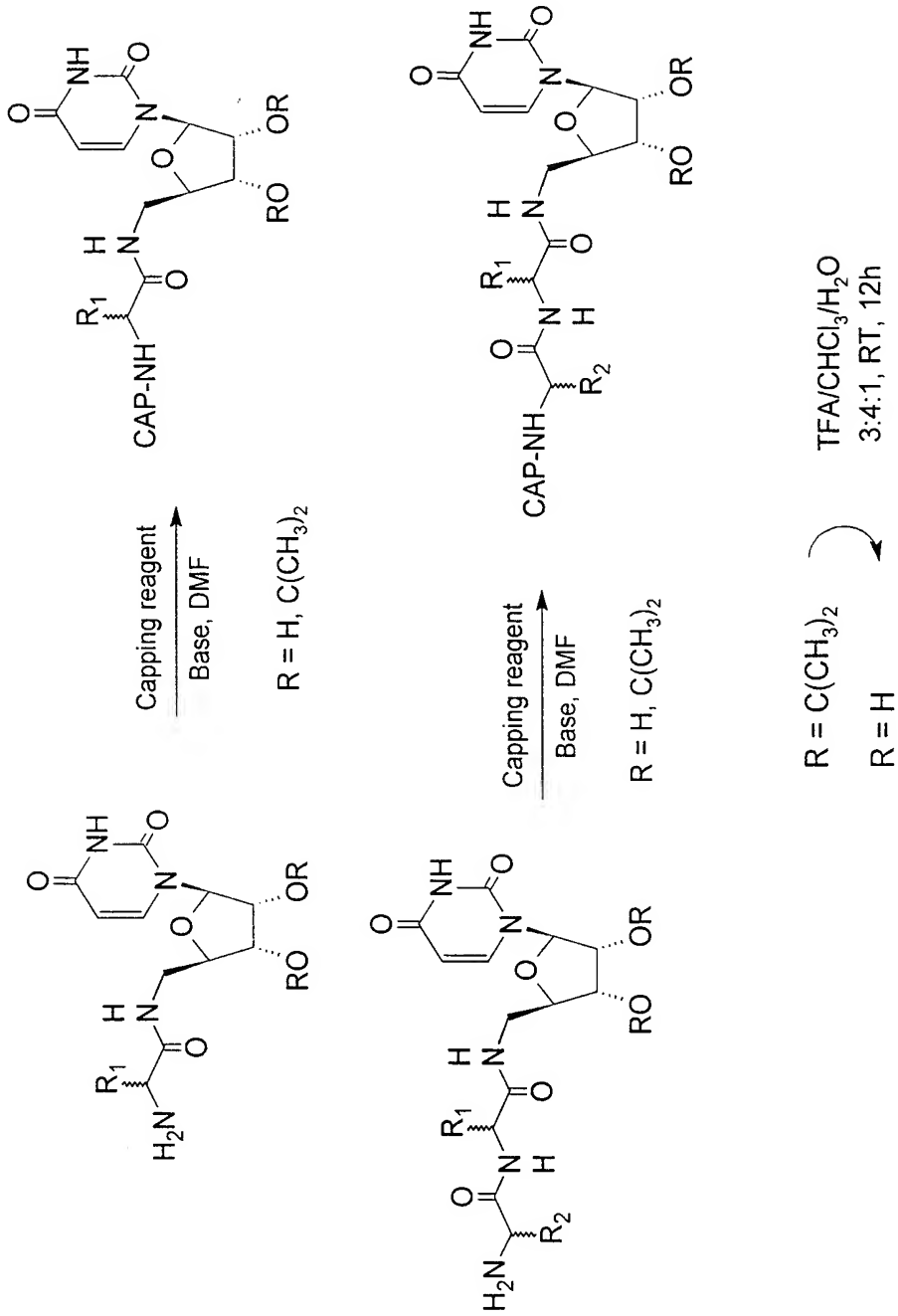
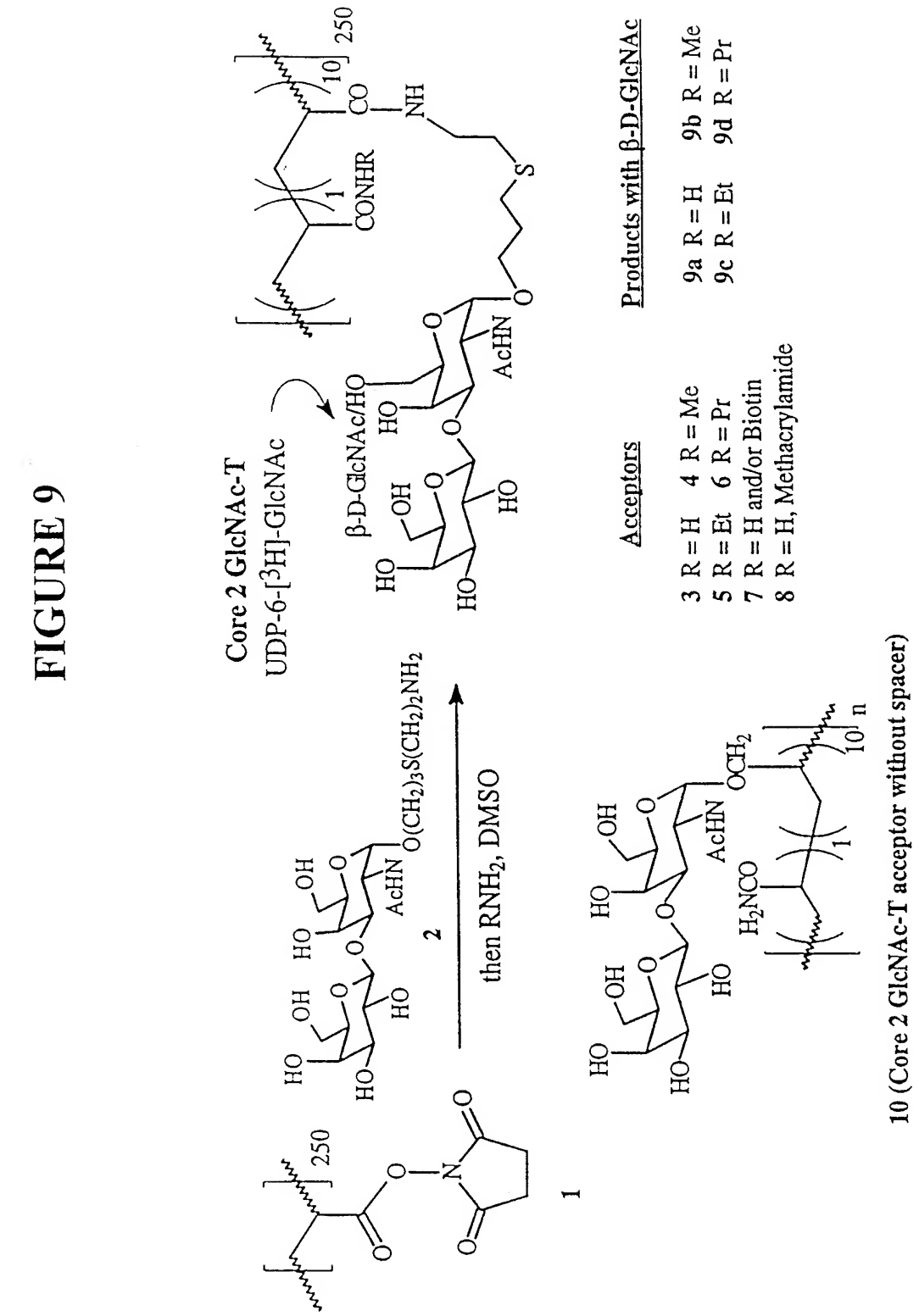

Capping reagent
Base, DMF
R = H, C(CH3)2
Capping reagent
Base, DMF
R = H, C(CH3)2
TFA/CHCl3/H2O
3:4:1, RT, 12h
R = C(CH3)2
R = H
CAP-NH
H2N

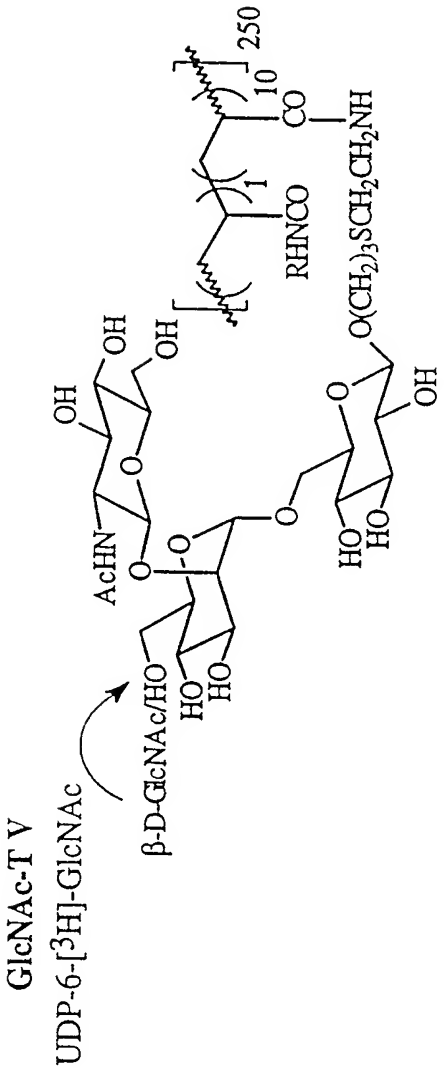
FIGURE 9



1

10 (Core 2 GlcNAc-T acceptor without spacer)

FIGURE 10



Acceptors

11 R = H 12 R = Me
13 R = Et 14 R = Pr

Products with β-D-GlcNAc

15 R = H 16 R = Me
17 R = Et 18 R = Pr

Figure 11

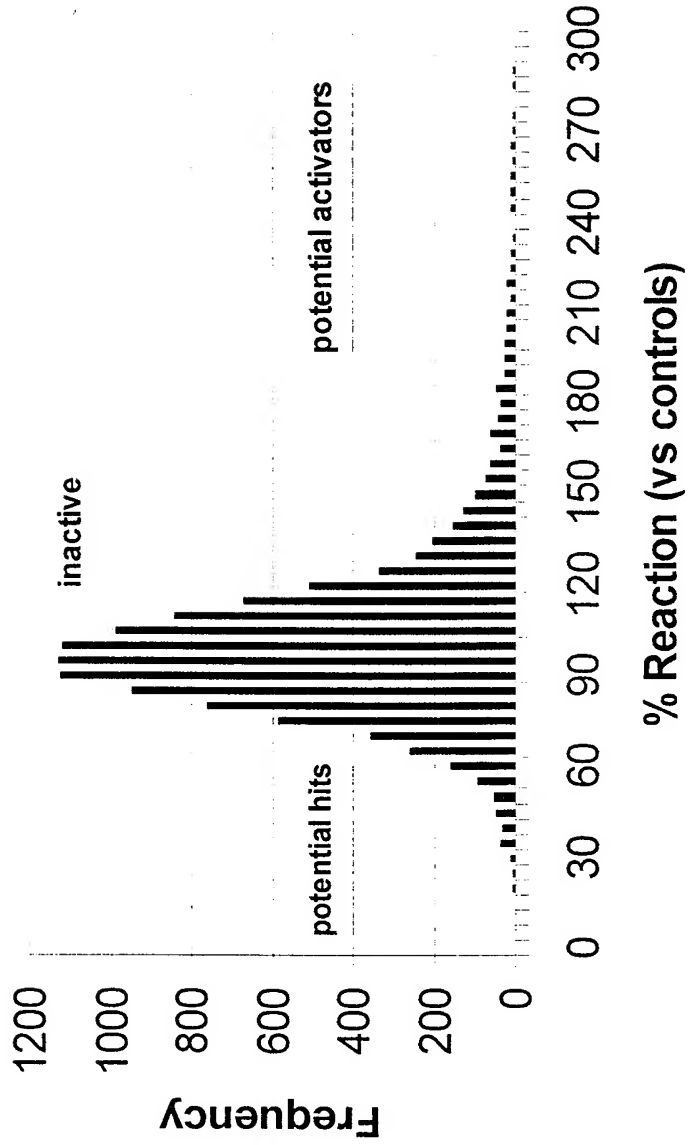

inactive
potential activators
potential hits
Frequency
1200
1000
800
600
400
200
0
0 30 60 90 120 150 180 210 240 270 300
% Reaction (vs controls)

Figure 12